IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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|---|---|
| Applicants: | |
| **Ian L. BROWN et al.** | Confirmation No.: 6243 |
| Serial No.: 10/009,023 | |
| Filed: April 12, 2002 | Group Art Unit: 1623 |
| For: STARCH SUB-TYPES AND LIPID METABOLISM | Examiner: L. Maier |

**DECLARATION OF IBRAHIM ABBAS PH.D.**
**PURSUANT TO 37 CFR § 1.132**

I, Ibrahim Abbas, residing at 6422 S. Heritage Place E, Centennial, CO 80112, hereby declare that:

1 I am a Senior Research Team Leader for Penford Food Ingredients, Co. and have been professionally employed as a carbohydrate chemist for twenty (20) years since receiving my Ph.D. in Agricultural Biochemistry and Nutrition in 1986. My qualifications and technical experience are set out in my *curriculum vitae*, a copy of which is attached as Appendix A.

2. I have read and understand the official actions from the U.S. Patent and Trademark Office (the "Patent Office") dated June 9, 2005 and March 10, 2006 (the "Office Actions"), which were issued in connection with U.S. Patent Application Serial No. 10/009,023. I also have reviewed and understand the patents and publications cited by the Examiner in the Office Action:

3. The Examiner raised an issue regarding the definiteness of the claim recitation of "amylase resistant starch." In particular, the Examiner argues that the specification does

not specify the method by which the amount of amylase resistant starch is measured and further that those of ordinary skill in the art would not have known how to determine the amount of amylase resistant starch according to the application claims.

4. Those of ordinary skill reading the teachings of the specification would be instructed to measure resistant starch levels by practice of the method of McCleary, Proc. 42nd RACI Cereal Chem. Conf. Christchurch NZ Ed. VJ Humphrey-Taylor pp 304-312 (1992) attached hereto as Exhibit A. Even if the specification had not taught use of this particular method (and it did), those of ordinary skill would have known to use this method because it was the method recognized by the Association of Official Analytical Chemists [AOAC].

5. Those of ordinary skill were taught by Applicants' specification that the McCleary method be used to determine resistant starch levels. Specifically, the disclosure at para. 0035 teaches:

> "[0035] As used in the specification, the term 'resistant starch' includes those forms defined as RS1, RS2, RS3 and RS4 as defined in Brown, McNaught and Molony (1995) Food Australia 47: 272-275."

Brown et al., Food Australia, (1995) (Exhibit B and Ref. C1) in turn taught that "resistant starch" was defined as "the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals" (pg. 272, col. 2 lines 8-14). Brown then cited Prosky et al., J. Assoc. Off. Anal. Chem. 71(5):1017 (1988) (Exhibit C) as providing "the officially accepted method of the Association of Analytical Chemists" for detecting resistant starch.

6. The Prosky method was considered the "gold standard" method for the measurement of total dietary fiber (TDF) and was designated AOAC Method 985.29. (See McCleary & Rossiter J. AOAC International, 87 No. 3 707-717 (2004) at. 707, col. 2 first full para.) (Exhibit D) and is the same as the McCleary method of Proc. 42nd RACI Cereal Chem. Conf. Christchurch NZ Ed. VJ Humphrey-Taylor pp 304-312 (1992).

7. For these reasons, one of ordinary skill in the art would have been directed by the teachings in the specification to use the method of McCleary for determining resistant starch content. Moreover, the references in the application to other publications which direct

use of the McCleary method merely reinforce the specific direction already provided in the disclosure to utilize the McCleary method.

8. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or document or any patent which may issue thereon.

Ibrahim Abbas Ph.D.

Date: August 23, 2006

## APPENDIX A

*Curriculum vitae*

**IBRAHIM R. ABBAS, PH.D.**

**7094 S. Revere Pkwy.**
**Centennial, CO 80112 Email:** iabbas@penx.com

**Work: 303-645-0167**
**Home: 720-488-0632**

**PROFESSIONAL EXPERIENCE:**

**Penford Food Ingredients Co., Centennial, Colorado 2003-Present**
**Sr. Research Team Leader**

- Design and conduct fundamental and applied research to develop new starch-based ingredient systems to meet specific market and application needs
- Leading a team of scientists to create new and innovative products for the food and pet industries
- Provide technical advice on various projects and customer solutions

**CARGILL, INC.**, Hammond, Indiana 1988 – 2003
**Manager-R&D** 2002 – 2003

Manage research efforts to develop customer-driven products as needs are identified.

- Developed three new products that resulted in over 4 million pounds of new business.
- Supervised development work to created production recipes for three tapioca-based products that led to a successful scale up to the plant with potential volume of 3-4 million pounds.
- Managed process improvement projects to decrease product losses that resulted in yield improvement of 2-3%.
- Led a team to evaluate and recommend product manufacturing between different plants reducing costs resulting in successful scale up trials of at least 10 products. Total volume impacted was at least 60 million pounds.
- Participated in a management integration team to facilitate transition of Cerestar, USA into Cargill resulting in a successful integration.

**CERESTAR, USA, INC.**, Hammond, Indiana 1996 – 2002
Acquired by Cargill, Inc. in 2002.

**Manager – Product Development** 2000 – 2002

Direct all groups with staffs of 10-12 in activities including product development, manufacturing and engineering, and sales and marketing support.

- Led product development projects for modified starches and pregelatinized starches resulting in a successful development of six new products and three million pounds of new business.
- Directed all product development and support activities for five different groups that resulted in a 20% improvement in sales of several specialty products.
- As a team leader, coordinated project activities via a cross-functional group consisting of sales, marketing, application, and product development resulting in a more timely completion of projects.
- Participated on a strategic planning team that set product development and commercial directions resulting in identifying more than 70 million pounds of targeted business in 25-30 different products,

**Manager – Center of Expertise Starch Technology** 1997 – 2000

Manage starch and refinery product development activities and process improvements.

- Developed one new glucose syrup to meet specific customer need resulting in more than 60 million pounds of new business.
- Led process and product improvement projects that resulted in at least 10% reduction in product rejection.
- Directed technology transfer projects that resulted in the transfer of at least five products to the US operations and about 3 million pounds of export business.

**Manager – Center of Expertise Starch Technology/ Application Center Food** 1996 – 1997

Manage all activities related to food applications and technical service.

- Led all activities related to new product development and line extensions that resulted in a successful completion of two new products.
- Refocused the food applications and technical service group that resulted in a more integrated R&D.
- Trained R&D staff on the new project management process resulting in a more integrated project portfolio to meet the global market needs.

**AMERICAN MAIZE-PRODUCTS COMPANY**, Hammond, Indiana 1988 – 1996

Acquired by Cerestar in 1996

**Manager-Product Development, Commercial Development** 1991 – 1996

Manage all efforts and line extensions for 14 products.

- Patented a process to produce an innovative product for food application that resulted in one million pounds of new business.
- Developed a new maltodextrin product that grew into over 10 million pounds of new business.
- Led Product Development Group to the development of twelve new products or line extensions resulting in 20% increase in sales of specialty products and an increase in market share from 16% to 20%.
- Initiated a process change to reduce cost and improve profitability of a high volume product that resulted in at least $200,000 reduction in production costs.
- Trained sales force, distributors, and new employees by giving at least 12 technical presentations improving productivity.

**Carbohydrate Chemist, Commercial Development** 1988 – 1991

- Developed and assisted in the commercialization of five new products resulting in a new business that has grown to at least 6 mm pounds per year.
- Developed and patented a product for use as a clouding agent in fruit-flavored beverages.

**IOWA STATE UNIVERSITY,** Ames, Iowa **1987 – 1988**
**Department Of Food Technology, Postdoctoral Research Associate**

**UNIVERSITY OF ARIZONA,** Tucson, Arizona **1979 – 1983**

**Dept. Of Nutrition and Food Science, Graduate Research Assistant 1985 – 1987**

**UNIVERSITY OF ARIZONA,** Tucson, Arizona
**Dept. of Family and Community Medicine, Research Assistant 1983 – 1985**

**UNIVERSITY OF ARIZONA,** Tucson, Arizona
**Department of Nutrition and Food Science, Graduate Student 1979 – 1983**

## EDUCATION

Ph.D. (1986) University of Arizona, Tucson, Arizona
Major: Agricultural Biochemistry and Nutrition; Minor: Food Science
Dissertation Title: Physicochemical Properties of Tepary Bean Starch

MBA (2000) Purdue University Calumet, Hammond, Indiana

## UNITED STATES PATENTS:

- US Patent # 4,971,828, issued November 20, 1990. Beverage Clouding Agent.
- US Patent # 5,139,809, issued August 18, 1992. Food Product Made from A Carotenoid-Free Corn Starch.
- US Patent # 5,192,576, issued March 9, 1993. Thick-Thin Retort Starch.

## PROFESSIONAL MEMBERSHIP:

Institute of Food Technologists
American Association of Cereal Chemists

## TECHNICAL PRESENTATIONS:

- AACC National Meeting, Dallas, Texas. Starch-Based Fat Sparing Ingredient. November 1990.
- AACC Central States Section Meeting, St. Louis, Missouri. Amalean I and Amalean II Instant Starch: Characteristics, Functionality and Applications. January 1991.
- Eastern Food Science Conference, Hunt Valley, Maryland. Amalean I and Amalean II Instant Starch: Properties, Functionality and Applications. October 1991.

- AACC Food Focus 97, Minneapolis, Minnesota. Modified Starches: Chemistry, Functions and Applications. April 1997.

- Technical Presentations to Customers: Kraft Foods, Campbell Soup, Heinz, General Mills, Pillsbury, Ross Labs, Quaker Oats, Continental Baking Company, Seafest Foods, Pfizer, Stouffer's, Hercules, Taste Maker, TIC Gums, etc.

**JOURNAL PUBLICATIONS:**

- **Abbas, I. R.**, A. M. Siddiqi, and S. J. Toma (1979). "Broad Bean Lipoxygenase: Partial Purification and Characterization." Food Chem. 4:269.

- Scheerens, J. C., A. M. Tinsley, **I. R. Abbas**, C. W. Weber, and J. W. Berry (1983). "The Nutritional Significance of Tepary Bean Consumption." Desert Plants 5:11.

- **Abbas, I. R.**, and J. W. Berry (1986). "Tepary Bean Starch: Part I. Physicochemical Properties." Starke 38:195.

- **Abbas, I. R.**, J. C. Scheerens, A. M. Tinsley, and J. W. Berry (1986). "Tepary Bean Starch: Part II. Rheological Properties and Suitability for Use in Foods." Starke 38:351.

- **Abbas, I. R.**, J. C. Scheerens, and J. W. Berry (1987). "Tepary Bean Starch: Part III. In Vitro Digestibility." Starke 39:280.

- Kabbara, S. A., **I. R. Abbas**, J. C. Scheerens, A. M. Tinsley, and J. W. Berry (1987). "Soaking and Cooking Parameters of Tepary Beans: Effects of Cooking Time and Cooking Temperature on the Hardness and Activity of Nutritional Antagonists." Plant Foods for Human Nutrition 36:295.

- Scheerens, J. C., M. J. Kopplin, **I. R. Abbas**, J. M. Nelson, A. C. Gathman, and J. W. Berry (1987). "Feasibility of Enzymatic Hydrolysis and Alcoholic Fermentation of Starch contained in Buffalo Gourd Roots." Biotech. and Bioeng. 29:436.

- White, P. J., **I. R. Abbas**, and L. A. Johnson (1989). "Freeze-Thaw Stability And Refrigerated-Storage Retrogradation of Starches." Starke 41:176.

- White, P., **I. Abbas**, L. Pollak, and L. Johnson (1990). "Intra- and Inter-Population Variability of Thermal Properties of Maize Starch." Cereal Chemistry 67(1): 70.

- D. Mauro, **I. Abbas**, and F. Orthoefer (2001). "Corn Starch Modification and uses" in "Corn: Chemistry and Technology." New book edition in press.

## APPENDIX B

Exhibit A: McCleary, Proc. 42nd RACI Cereal Chem. Conf. Christchurch NZ Ed. VJ Humphrey-Taylor pp 304-312 (1992).

Exhibit B & Ref. C1:: Brown et al., Food Australia, (1995).

Exhibit C: Prosky et al., J. Assoc. Off. Anal. Chem. 71(5):1017 (1988).

Exhibit D: McCleary & Rossiter J. AOAC International, 87 No. 3 707-717 (2004).

# A RAPID PROCEDURE FOR TOTAL STARCH MEASUREMENT IN CEREAL GRAINS AND PRODUCTS

B.V. McCleary*, T.S. Gibson* and V. Solah*

*Megazyme (Aust) Pty Ltd
6 Altona Place, North Rocks (Sydney), NSW 2151, Australia
* Biological and Chemical Research Institute
NSW Agriculture, PMB 10, Rydalmere, NSW, 2116, Australia

## INTRODUCTION

Total starch determination methods in common use can be broadly grouped into acid hydrolysis or enzymic procedures (Anon, 1987; Mitchell, 1990). Acid hydrolysis procedures can only be applied to pure starch samples and thus have limited application. Enzymic procedures vary mainly in the procedures employed to remove interfering sugars (e.g. glucose and maltodextrins) and to gelatinise the starch, and in the enzymes used to liquefy the starch and convert oligosaccharide fragments to glucose. Glucose is generally measured using glucose oxidase/peroxidase or hexokinase/glucose 6-phosphate dehydrogenase based procedures (Batey, 1982). Glucose and dextrins have been removed from samples being analysed by extraction of the samples with boiling aqueous ethanol or 75% propan-2-ol (Karkalas, 1985). Interference from glucose can also be removed by a borohydride reduction step (Henry, Blakeney and Lance, 1990). Starch gelatinisation has been effected by autoclaving aqueous starch slurries (AACC Method 76-11) or by dissolving the starch in NaOH or KOH solutions (Karkalis, 1985) or in dimethyl sulphoxide (DMSO) (Englyst and Cummings, 1984, 1988) at elevated temperatures. Starch liquefaction and/or dextrinisation has involved treatment of gelatinised starch with amyloglucosidase (AACC Method 76-11) or pancreatic (Englyst and Cummings, 1984) or thermostable (Baur and Alexander, 1979; Theander and Aman, 1979; Batey, 1982; Aman and Hesselman, 1984) $\alpha$-amylases. In some of these formats, the starch has been gelatinised in the presence of thermostable $\alpha$-amylase. Pullulanase has been used to improve the degree of starch hydrolysis before amyloglucosidase hydrolysis of dextrins to glucose (Englyst and Cummings, 1984). The amyloglucosidase incubation step has been performed both on the total hydrolysate and on aliquots removed after adjustment of the hydrolysate to a set volume.

The aim of the currently reported research was to evaluate the various enzymic formats for measurement of total starch and to define a procedure which could be used on a wide range of samples including high amylose and high amylopectin starches, chemically modified starches, cereal flours and cereal based food products. A second aim was to develop a format in which, in the first phase, the starch is quantitatively solubilised with minimum release of free glucose.

## MATERIALS AND METHODS

Cereal flours and starch samples were obtained from commercial suppliers. Cereal products (e.g. cornflakes, spaghetti) were milled to pass a 0.5 mm screen using a Fritsch centrifugal mill (Fritsch GmbH, D-6580 Idar-Oberstein, Germany) with a 12-tooth rotor. Thermostable $\alpha$-amylase and pullulanase were obtained from Novo Nordisk and Rhone-Poulenc, Brewing and Food Group. These were purified (where necessary) by ion exchange and gel permeation chromatographic procedures. High purity amyloglucosidase, bacterial $\beta$-amylase and glucose oxidase/peroxidase glucose determination reagent (GOPOD) were from Megazyme (Aust.) Pty Ltd. All other chemicals were laboratory grade reagents.

$\alpha$-Amylase activity was measured with Amylazyme tablets at 50°C in MOPS buffer (0.1 M, pH 7.0) and converted to Units of activity on soluble starch via a standard curve (McCleary, 1991). Pullulanase was assayed with Limit-Dextrizyme tablets and converted via a standard curve to Units of activity on pullulan (Nelson/Somogyi method, 50°C, pH 5.0) (McCleary, 1992). $\beta$-amylase was assayed on soluble starch (10 mg/mL) at pH 5 and 50°C.

Starch content was measured using AACC Method 76-11, the method of Blakeney (personal communication: a modification of Henry, Blakeney and Lance, 1990) except that the samples were not "ball-milled" as recommended by Blakeney. Ball milling would yield higher values for some of the samples analysed. Acid hydrolysis was performed by incubating starch sample (100 mg) with 2 N trifluoro acetic acid (2 mL) at 120°C for 60 min. The solution was transferred to a 100 mL volumetric flask and adjusted to volume with 50 mM sodium acetate buffer (pH 4.5).

**Total starch assay : Format 1**

Starch or flour sample (100±10 mg, accurately weighed) is weighed into a glass test-tube (14x120 mm) and the tube is tapped so that all sample falls to the bottom of the tube. The sample is wet with aqueous ethanol (0.2 mL, 50% v/v) and stirred on a vortex mixer. DMSO (1 ml) is added using a positive displacement dispenser, while the tube is being stirred. The tube is immediately placed in a vigorously boiling water bath and cooked for 2 min. After 2 min, an aliquot (2 mL) of thermostable α-amylase (80 Units) in 50 mM MOPS buffer (pH 7.0) is added to the tube (while still in the boiling water bath) and the mixture is incubated for 1 min, removed from the bath and vigorously mixed on a vortex stirrer for 10 sec. The tube is returned to the boiling water bath for a further 1 min. After exactly 2 min (from time of addition of the α-amylase), remove the tube from the bath and add an aliquot (4.0 mL) of 100 mM sodium acetate buffer (pH 4.5) containing pullulanase (10 Units) and β-amylase (100 Units). Mix the tube contents vigorously for 10-20 sec and incubate the tubes at 50°C for 1 hour. Stir the tube contents 2-3 times during this incubation period.

Quantitatively transfer the tube contents to a 100 mL volumetric flask (washing the tube with water from a wash bottle) and adjust the volume to the mark. Aliquots (0.1 mL) in triplicate are transferred to the bottom of glass test tubes. To two of these (reaction tubes) is added amyloglucosidase (0.1 mL, 2 Units) in sodium acetate buffer (100 mM, pH 4.5) and to the third (the "blank") is added sodium acetate buffer (0.1 mL, 100 mM, pH 4.5). The tubes are incubated at 50°C for 10 min and then treated with GOPOD reagent (3.0 mL) and incubated at 50°C for 20 min. The absorbance of the reaction solution and reaction blank is measured (510 nm) against a reagent blank. The reagent blank is prepared by incubating GOPOD reagent (3.0 mL) with acetate buffer (0.2 mL, 100 mM, pH 4.5) at 50°C concurrent with the reaction solutions. Glucose standards (50 and 100 μg in 0.2 mL) are incubated concurrently with GOPOD reagent (3.0 mL).

**Total starch assay : Format 2**

Starch or flour sample (100±10 mg, accurately weighed) is weighed into a glass test-tube and wet with aqueous ethanol (0.2 mL, 50% v/v) and stirred, as in Format 1. The sample is then treated with an aliquot (1.0 mL) of thermostable α-amylase (80 Units) in MOPS buffer (50 mM, pH 7.0), stirred vigorously and added to a vigorously boiling water bath. After 1 min, the tube is removed and the contents stirred vigorously (on a vortex mixer), and the tube returned to the boiling water bath for a further 2 min (total of 3 min). The tube is removed from the boiling water bath and treated with acetate buffer (4 mL) containing pullulanase and β-amylase as in Format 1 and the assay completed as in Format 1.

**Total starch assay : Format 3 (Susceptible Starch)**

In this procedure, the sample is weighed and wet with aqueous ethanol, as in Formats 1 and 2. The sample is then treated with an aliquot (1.0 mL) of thermostable α-amylase (80 Units), stirred vigorously and placed in a vigorously boiling water bath. After 1 min, the tube is removed from the bath, stirred vigorously and returned to the boiling water bath for a further 10 min. The tubes are then treated with cold, dilute sulphuric acid (2 mL, 0.2%) and placed in a rack in water at room temperature (approx. 22°C). The volume is adjusted to 100 mL and aliquots (0.1 mL) in triplicate are removed for glucose determination after amyloglucosidase treatment (as in Format 1).

## CALCULATIONS:

$$\text{Starch (as is) (Total or Susceptible)} = \Delta E \times F \times 1000 \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180}$$

where:

$\Delta E$ = absorbance (reaction) read against the reagent blank

$F$ = $\frac{100\ (\mu g \text{ of glucose})}{\text{absorbance for } 100\ \mu g \text{ of glucose}}$ (conversion from absorbance to $\mu g$)

1000 = volume correction (0.1 mL taken from 100 mL)

$\frac{1}{1000}$ = conversion from micrograms to milligrams

$\frac{100}{W}$ = factor to express "total starch" as a percentage of flour weight

$W$ = the weight in milligrams ("as is" basis) of the flour analysed

$\frac{162}{180}$ = adjustment from free glucose to anhydro glucose (as occurs in starch).

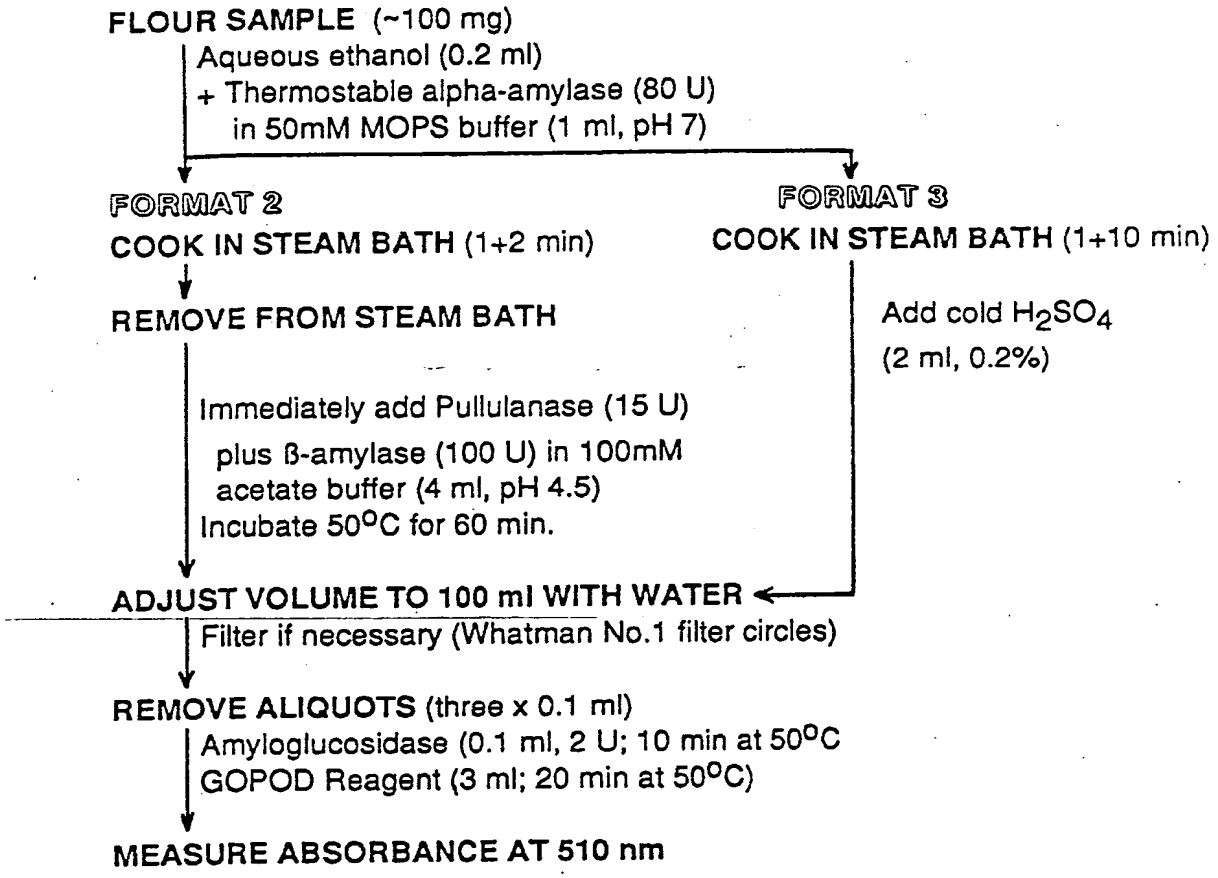
## RESULTS AND DISCUSSION

The aim of this research was to develop a procedure which could be used to measure total starch in a wide range of plant materials and which would also give some indication of the amount of free glucose in the sample. In the initial approach to this problem, starch was gelatinised in a boiling water bath in the presence of the minimum level of thermostable $\alpha$-amylase required to give starch

**Table 1. Measurement of the starch content of cereal products : a comparison of methods.**

| Sample | Moisture (%) | Assay Formats: Starch Content (%dw) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Format 1 | Format 2 | Format 3 | AACC | Blakeney | Acid Hydrolysis |
| Waxy Maize Starch | 12.0 | 99.5 | 99.5 | 96.7 | 82.1 | 98.9 | 94.5 |
| High Amylose Maize Starch | 13.0 | 99.0 | 79.0 | 71.0 | 67.7 | 86.6 | 91.0 |
| Cross Linked Starch 1 | 13.0 | 99.0 | 89.5 | 81.6 | 56.1 | 91.3 | 91.2 |
| Cross Linked Starch 2 | 13.0 | 98.7 | 98.6 | 58.4 | 76.0 | 94.4 | 93.0 |
| ACS Soluble Starch | 11.0 | 99.0 | 99.0 | 96.2 | 95.2 | 98.0 | 96.0 |
| Spagetti | 12.0 | 82.0 | 82.0 | 82.0 | 76.1 | 76.5 | 82.0 |
| CornFlakes | 6.8 | 81.8 | 81.8 | 76.4 | 72.8 | 73.1 | 77.6 |

solubilisation. The aim was to achieve starch solubilisation with minimum or nil release of free glucose. This approach is detailed as Format 3 and is represented schematically in Scheme 1. To obtain maximal "apparent starch", an incubation time of 1 and 10 min with α-amylase at ~95°C was required. Under these conditions, the amount of free glucose in the reaction blank (no amyloglucosidase treatment) increased to about 0.07–0.08 (i.e. to about 7–8% of the reaction value) (refer to Fig. 1). This blank value could be reduced by reducing the time of incubation with α-amylase, but this resulted in a concurrent decrease in measured total starch.




**Scheme 1. Measurement of "apparent" total starch : Assay Formats 2 and 3.**

On application of assay Format 3 to a range of samples including high amylose maize starch (Table 1), it was apparent that the procedure was not measuring total starch. For high amylose maize starch, apparent starch values of only 71% were obtained. Similar values were obtained with AACC Method 76–11.

Figure 1. Effect of incubation time with thermostable α–amylase on "apparent" total starch values (shown as absorbance values) and blank absorbance values.

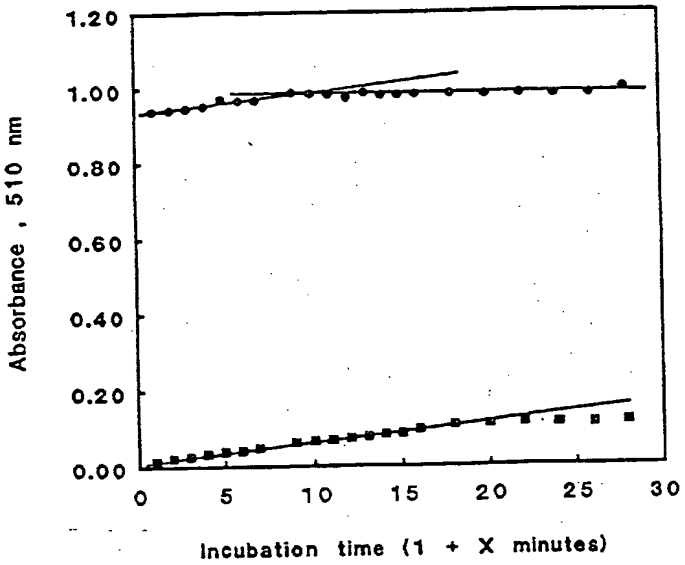



**Table 2. Measurement of starch content: effect of different assay formats on "total" starch values and blank absorbance values.**

| Sample | Assay Formats | | | | | |
|---|---|---|---|---|---|---|
| | Format 1 | | Format 2 | | Format 3 | |
| | Blank Absorbance | Starch (%) | Blank Absorbance | Starch (%) | Blank Absorbance | Starch (%) |
| Waxy Maize Starch | 0.039 | 99.5 | 0.038 | 99.5 | 0.070 | 96.7 |
| High Amylose Maize Starch | 0.034 | 99.0 | 0.040 | 79.0 | 0.073 | 71.0 |
| ACS Soluble Starch | 0.040 | 99.0 | 0.030 | 99.0 | 0.081 | 96.2 |

For difficult to hydrolyse samples, higher apparent starch values can be obtained by using higher levels of α–amylase and incubating for longer periods, e.g. the Blakeney method (~4000 U for 60 min) (a modification of the method of Henry, Blakeney and Lance, 1990), as shown in Table 2. However, with this modification, the blank absorbance values were very high due to glucose released from the starch fragments by the α–amylase. An alternative approach is to use pullulanase (which does not produce free glucose) to debranch the starch fragments; together with excess β–amylase, to rapidly hydrolyse these fragments to maltose (which is not a substrate for the α–amylase). The principle of this assay format (Format 2) is shown in Scheme 1, and results obtained are shown in Tables 1 and 2. It is apparent that this format gives higher "apparent starch" values for a range of samples, with significantly reduced blank absorbance values. However, the starch values obtained for some samples is still less than that obtained using the Blakeney procedure.

**FLOUR SAMPLE** (~100 mg)
↓ Aqueous ethanol (0.2 ml)
DMSO (1 ml)

**COOK IN STEAM BATH** (2 min)
↓ Thermostable alpha-amylase (80 U)
in 50mM MOPS buffer (2 ml, pH 7), (2 x 1 min at 95°C).

**REMOVE FROM STEAM BATH**
↓ Immediately add pullulanase (15 U) plus ß-amylase (100 U)
in 100mM acetate buffer (4 ml, pH 4.5).
Incubate at 50°C (60 min).

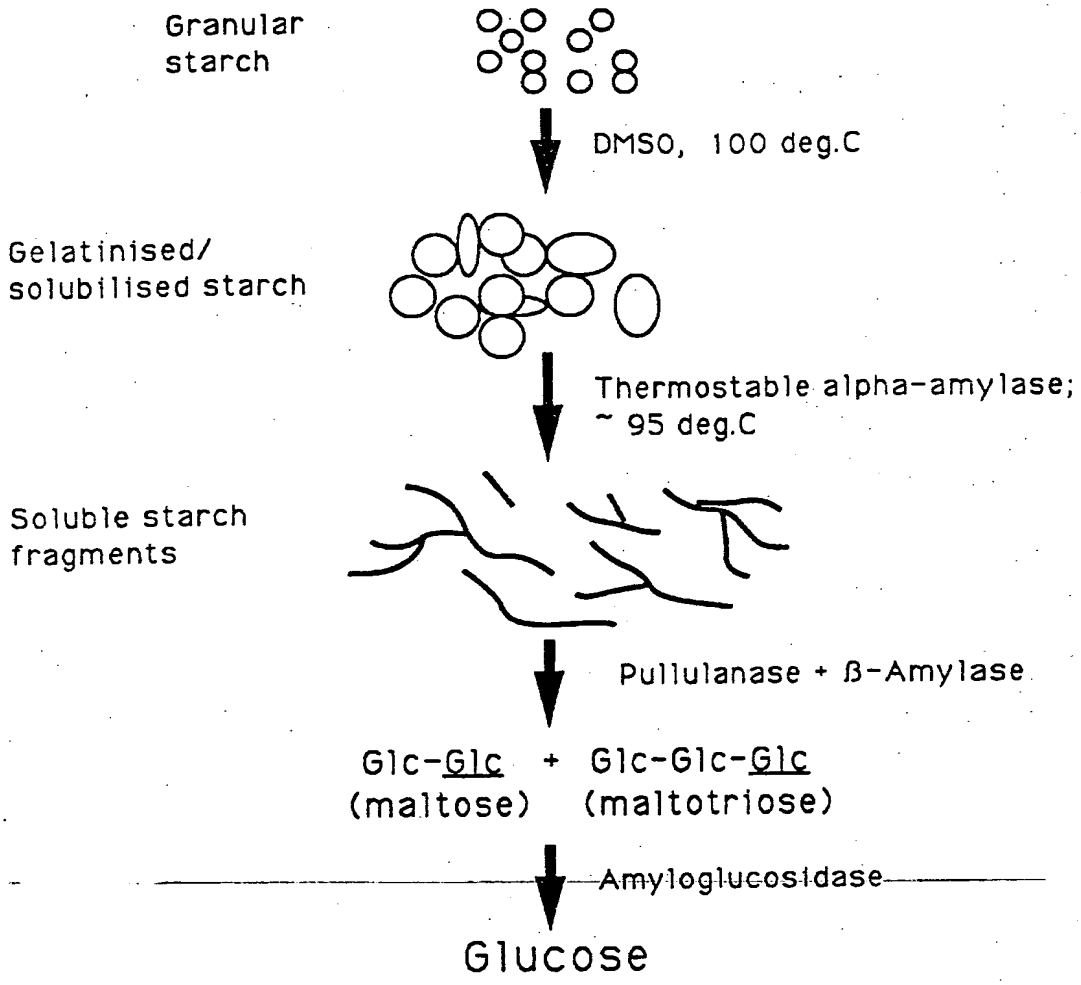
**TRANSFER TO 100ml VOLUMETRIC FLASK AND ADJUST VOLUME TO THE MARK WITH WATER**
↓ Filter if necessary (Whatman No.1 filter circles)

**REMOVE ALIQUOTS** (three x 0.1 ml)
↓ Amyloglucosidase (0.1ml, 2 U; 10 min at 50°C)
GOPOD Reagent (3 ml; 20 min at 50°C)

**MEASURE ABSORBANCE AT 510 nm**

**Scheme 2. Measurement of total starch: Assay Format 1.**

It became apparent that with certain samples, and in particular, high amylose maize starch, the major problem was associated with the initial gelatinisation and solubilisation of the sample. We thus evaluated several solvents including sodium hydroxide and potassium hydroxide solutions and dimethyl sulphoxide (DMSO). Of these, we found DMSO to be superior by far. The assay format incorporating DMSO is outlined in Schemes 2 and 3. The starch is gelatinised in DMSO at 100°C and then solubilised by slight treatment with thermostable α–amylase. The homogenate is then treated with pullulanase/β–amylase for 60 min at 50°C. Using this format, quantitative yields of starch could be obtained for the full range of samples analysed except for one sample (Sigma Amylose cat. no. A7043). The values obtained were the same as, or greater than those obtained using any of the other procedures studied, including acid hydrolysis procedures (except for Sigma amylose).




**Scheme 3. Pictorial representation of the reactions involved in the measurement of total starch using Format 1.**

The reproducibility of the starch assay Formats 1-3 for three starch samples is shown in Table 3. Each of the methods is very reproducible (with similar standard deviations). Format 1 gives a measure of total starch whereas Format 3 measures enzyme "susceptible" starch. An indication of the level of resistant starch in a sample can be obtained by subtracting values obtained by these assay formats:

Thus:

Resistant Starch = Starch (Format 1) (total starch) − Starch (Format 3) (susceptible starch)

Table 3. Starch determination: reproducibility of assay Formats 1–3.

| Sample | Moisture content | Starch Content% (Dry Wt basis) | | |
|---|---|---|---|---|
| | | Format 1 | Format 2 | Format 3 |
| Waxy Maize Starch | 12.0 | 98.0 ± 2.0 | 98.0 ± 2.0 | 95.0 ± 2.2 |
| High amylose Maize Starch | 13.0 | 97.0 ± 2.0 | 78.0 ± 2.0 | 70.0 ± 2.1 |
| RACI Wheat Flour | 14.2 | 70.0 ± 2.0 | 68.0 ± 2.1 | 65.0 ± 2.0 |

* n = 6

## CONCLUSIONS

In the current paper we describe assay formats which can be used to measure total starch (Format 1) and enzyme "susceptible" starch (Format 3) in a wide range of samples. In assay Format 1, DMSO is used to solubilise the starch. This solvent should be handled with care, and if possible avoided. For most of the cereal flours analysed, the values obtained with Format 2 (using water instead of DMSO) were the same as those with Format 1.

We recommend that in initial studies, Format 1 be employed. When the procedure is mastered, Format 2 should be evaluated. If both methods yield the same values, Format 2 could be used with confidence to measure total starch.

The reaction blank values obtained in assay Formats 1 and 2 are about 0.03–0.04. These absorbance values are obtained with pure starch samples and thus are due to glucose released during the α-amylase hydrolysis step. If reaction blank values are higher than this, then the sample must contain free glucose. This free glucose should be measured separately and subtracted from the total starch value.

The assay Formats described in this paper, and the enzymes and reagents required to perform these analyses are available in kit form (Total Starch Assay Kit) from Megazyme (Aust.) Pty Ltd.

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AIFST 1995 FOOD INDUSTRY INNOVATION ADDRESS

EXHIBIT B

# *Hi-maize*™: new directions in starch technology and nutrition

**I. L. Brown, K.J. McNaught and E. Moloney**

Maize or corn is the sixth most valuable crop grown in Australia although there are only some 200,000 t harvested each year (Anon 1991) mainly from Queensland and NSW. Starch Australasia Ltd, a wholly owned subsidiary of Goodman Fielder Ingredients Ltd, is the only company in Australia which is involved in the further processing of maize to extract the starch for food and industrial use. Starch Australasia Ltd is also involved in the manufacture of products from wheat with the combined operations processing some 100,000 t of domestically sourced grain, employing 375 people in Australia and New Zealand, providing expenditure on research and development of more than A$1.2 m per year and with annual sales revenue of approximately A$110 m.

Starch Australasia Ltd is an Australian owned company with two manufacturing sites in Australia and one in New Zealand. At Tamworth in NSW wheat flour is extracted to provide starch, starch-derived products and gluten. The second site, at Lane Cove in NSW, is the only Australian manufacturing operation for wet milling and modifying starch from regular, waxy and high amylose maize. This mill has recently celebrated 100 years of operation. The facility situated in Auckland, New Zealand trades under the name New Zealand Starch Products Ltd and produces ingredients based on native and modified regular maize starch. In recent years some A$30 m has been spent undertaking major capital redevelopment of Starch Australasia Ltd facilities in order to improve product quality, increase production capabilities and allow for the introduction of innovative products.

Starch is the main storage carbohydrate found in higher plants including cereals such as maize. The starch granule provides an economical means of storing carbohydrate in an insoluble and densely packed manner (Imberty & others, 1991). The synthesis, composition and development of starch granules are influenced by a wide range of genetic and environmental factors (Shannon & Garwood 1984, Badenhuizen 1969). Maize varieties must be bred and adapted to the agronomic conditions of the region where they will be grown. Starch Australasia Ltd has actively encouraged and participated in the breeding and adaption of waxy and high amylose maize varieties for Australian conditions.

Once harvested the starch is separated from the other components in the kernel by using a conventional wet milling procedure. The kernel is steeped in a dilute solution of sulphurous and lactic acids to soften the pericarp and facilitate the sequential separation of the germ, fibre and protein (May 1987). The maize starch recovered is dried, bagged and tested prior to use by customers.

The importance of starch in the diet and its potential to contribute in many positive ways to people's health and well-being has been widely reported during the past decade. One area of interest identified was that some starch when consumed resists enzymatic digestion and acts like dietary fibre. The term "resistant starch" is used to describe this type of starch. Resistant starch has been defined by EURESTA (European Food-Linked Agro-Industrial Research - Concerted Action on Resistant Starch 1991) as "the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals". In this regard resistant starch acts like other dietary fibres and is one of those substances detected by the officially accepted method of the Association of Analytical Chemists (Prosky & others, 1988) along with non-starch polysaccharides and lignin. A number of factors may cause starch to be resistant to digestion, including the size of the starch-containing fragments, eg in kibbled grains, the structure and conformation of intact starch granules, the formation of retrograded crystallites as a result of processing, and chemical modification (Table 1). Resistant starch is found in many types of food commonly consumed (Crawford 1987).

## Development of *Hi-maize*™

Since 1975 Starch Australasia Ltd has been engaged in a research and commercial program to grow and develop uses for high amylose maize starch. Using germ plasm obtained from overseas and conventional breeding tech-

**Table 1. Nutritional classification of starches.**

| Type of starch | | Example of occurrence | Probable digestion in small intestine |
|---|---|---|---|
| Rapidly digestible | | Freshly cooked starchy food | Rapid |
| Slowly digestible | | Most raw cereals | Slowly but completely |
| RESISTANT STARCH | | | |
| RS1 | Physically inaccessible | Partly milled grains and seeds | Resistant |
| RS2 | Resistant granules | Raw potato, green banana, some legumes and *Hi-maize*™ | Resistant |
| RS3 | Retrograded starch | Cooked and cooled potato, bread and cornflakes | Resistant |
| RS4 | Chemically modified starch | Starch ethers, esters and crossbonded | Resistant |

Adapted from Englyst & others (1992).

*Ian Brown is Principal Scientist, Goodman Fielder Ingredients, 45-47 Green St, Botany, NSW 2019; Ken McNaught is R&D Manager, Starch Australasia Ltd, 170 Epping Rd, Lane Cove, NSW 2066; Eric Moloney is principal of High Yield Seed Co, No. 9 Bristane St, Tamworth, NSW 2340.*

niques, a population hybrid containing 55 to 65% amylose was bred and adapted to Australian conditions (McWhirter & Dunn 1986). Initial interest was on the use of this high amylose maize starch in retorted products. Towards the late 1980s interest expanded to encompass biodegradable polymer films, controlled release matrices and, for nutritional and functional reasons, use in foods.

A systematic examination of the structure and physical properties of the starches obtained from existing and experimental Australian maize varieties was conducted by Ian Brown during 1990 to 1993 in association with the research required for a Master of Science degree at the University of New England. The work was principally conducted at the Research and Analytical Laboratories of Goodman Fielder Ingredients Ltd in Tamworth, NSW. Based on the research data a number of relationships were observed in respect to paste viscosity, granule size, molecular weight and detectable levels of dietary fibre and resistant starch.

Starch is composed of two major components. Both are glucose polymers, amylose being essentially linear and amylopectin having a highly branched structure. The level of amylose in the high amylose maize varieties appeared to have a direct relationship to the level of resistant starch and dietary fibre in the starch (Table 2) (Brown 1993).

**Table 2. Dietary fibre Content of Australian maize starches.**

| | Amylose content* (%) | Dietary fibre content* (%) |
|---|---|---|
| Waxy maize | 0 | 0 |
| Regular maize | 28 | 0 |
| High amylose maize varieties | 55 | 5.9 |
| | 64 | 8.8 |
| | 73 | 21.0 |
| | 76 | 27.5 |
| | 85 | 33.4 |

* dry solids basis
From Brown & others (1993)

One cultivar, bred by Eric Moloney, provided a starch that had good agronomic performance and a uniquely high level of resistant starch/dietary fibre in combination with a range of properties of potential commercial interest:

- Natural
- Source of dietary fibre/resistant starch
- White/invisible
- Survives most normal processing conditions
- Provides functional properties for foods
- Possible nutritional benefits
- Opportunity for innovation

The opportunities for this starch to be included in a wide of range foods led to the formulation of a plan to market this product.

A Goodman Fielder taskforce involving I Brown, K McNaught, F Lee, R Ganly, J Gould, A O'Brien, W Morgan and B Cox was formed to address the technical, legal and nutritional issues relating to the release of this novel ingredient. Alan O'Brien was given the task of bringing the product to market and developing a trademark that could be easily identified. *Hi-maize*™ was commercially released in September 1993 by the Minister for Primary Industry, the Rt. Hon. Simon Crean. *Hi-maize*™ was the first commercially available resistant starch product in the world and was the result of Australian agricultural research, manufacturing expertise and food technology.

*Hi-maize*™ is rich in resistant starch and is a natural white source of dietary fibre that can be added to foods such as bread, buns, breakfast cereals, pasta, biscuits, extruded foods and snacks without adversely affecting their organoleptic properties. The ability of *Hi-maize*™ not to fully hydrate and gelatinise until heated to more than 150°C means that the granular structure of the starch can be maintained under most normal food processing conditions. *Hi-maize*™ contributes resistant starch and dietary fibre to foods without the necessity for artificial or chemical pretreatments, as in the case of retrograded or chemically modified resistant starches. *Hi-maize*™ contributes functionally to foods by increasing moisture retention, improving expansion after extrusion and by providing a barrier film that retards the uptake of fats in snack foods (Brown 1994). *Hi-maize*™ and foods that include it are the subject of three international provisional patents.

## Nutritional properties

Nutritional studies conducted by the University of Sydney, the University of Melbourne, Deakin University and the CSIRO Division of Human Nutrition have indicated the potential of *Hi-maize*™ to impact positively on the health and well-being of people. The physiological effects of *Hi-maize*™ appear to occur in both the small and the large bowel. Since *Hi-maize*™ is only partially digested in the small intestine its inclusion in foods such as bread (Table 3) lowers their glycemic index. This means that glucose is released at a slower rate into the blood stream than from foods which contain rapidly digested starch.

Studies at the University of Sydney indicated that consumption of *Hi-maize*™, instead of readily digested low amylopectin or waxy maize starch, significantly reduces development of insulin resistance in rats when eaten over an 8 week period (Byrnes & others 1994). These observations may have important implications for people susceptible to the development of diabetes or those treating a diabetic condition.

*Hi-maize*™ has been shown to arrive in the large bowel in both animal (Brown & others 1994) and human studies (Muir & others 1995). Upon arrival in the large bowel the *Hi-maize*™ acts as a fermentable substrate for the native microflora and produces a range of effects that are believed to improve bowel health. These effects include a

**Table 3. Glycemic index of bread.**

| | Glycemic index |
|---|---|
| Commercial white bread | 100 |
| Containing 5% *Hi-maize*™ | 96 ± 16 |
| Containing 10% *Hi-maize*™ | 74 ± 14 |
| Containing 20% *Hi-maize*™ | 53 ± 12 |
| Wholemeal bread | 84 ± 23 |

From Muir & others (1994a)

lowering of fecal pH, an increase in the levels of short chain fatty acids, in particular propionate (Topping & others 1995) and butyrate (Muir & others 1994b), increased bowel action, through a mild laxative effect, and a reduction in the level of secondary bile acids in the feces, in particular deoxycholate (Clifton & others 1995). The inclusion of *Hi-maize*™ in the diet of pigs was found to increase the length of the large bowel. This may assist in individuals where the size of the bowel may not have developed to its potential (Topping & others 1995) . The importance of starch in our diets and its potential to improve public health has stimulated considerable interest in the scientific community (Muir & others 1993, Annison & Topping 1994). Nutritional research concerning *Hi-maize*™ is continuing both in Australia and overseas, with particular emphasis on the fermentation of the *Hi-maize*™ in the large bowel.

### *Hi-maize*™ in bread

Quality Bakers Australia Ltd were able to use *Hi-maize*™ to produce a white bread with a dietary fibre content higher than that of multigrain bread but with the taste characteristics of traditional white bread (Table 4). This bread *Wonder White*™ was released in April 1994. Health authorities in Australia recommend that Australians should consume more dietary fibre and increase their intake of bread. However some people, particularly children, refrain from consuming the high fibre multigrain and wholemeal breads because of their preference for white bread. Conventional types of dietary fibre, such as wheat bran, have always coloured the bread, changed the texture by making it more fibrous and chewy or decreased the softness and volume of the loaf. *Hi-maize*™ allowed for the first time manufacture of a soft high fibre white bread with excellent keeping qualities.

*Wonder White* by Quality Bakers Australia Ltd.

The acceptance of *Wonder White*™ in Australia has been remarkable; 20 weeks after launch it had captured 12% of the Australian white bread market and increased the size of the white bread market by 8%. More importantly *Wonder White*™ has encouraged more people to eat bread in accordance with dietary guidelines. For the first time in many years total bread consumption in Australia increased by 1.4% during the period since the launch.

The success of *Wonder White*™ has already led to the release of a similar high fibre white bread in New Zealand called *Nature's Fresh Fibre White*™ by Quality Bakers New Zealand Ltd, and further product launches in other countries are expected in the near future.

### Innovative foods

*Hi-maize*™ has enabled the development of innovative food products such as the breakfast cereal *Wheat Bites* released by The Uncle Tobys Co in 1994. In this breakfast cereal *Hi-maize*™ acts in a multifunctional manner. Not only does it provide a dietary fibre level in *Wheat Bites* of 13.1%, but this dietary fibre gives the extruded cereal a light, well-expanded texture and excellent crunch. Owing to the film forming properties of the *Hi-maize*™ the *Wheat Bites* retain this crisp texture in the bowl after the milk has been added. The Uncle Tobys Co are continuing to investigate other innovative ways of utilising *Hi-maize*™.

*Hi-maize*™ can be included as a source of dietary fibre in foods for people with specialist requirements such as those who suffer from coeliac disease. This group are sensitive to gluten and have difficulty in obtaining foods which contain dietary fibre but do not aggravate their condition. Conventional dietary fibres, such as wheat bran, contain the proteins to which coeliacs


*Natures Fresh Fibre White* by Quality Bakers New Zealand Ltd.

**Table 4. Dietary fibre content of commercial bread.**

| | Dietary fibre level (g/per 100 g as consumed) |
|---|---|
| *Wonder White* | 5.6 |
| Multigrain bread* | 4.8 |
| Traditional White Bread* | 2.9 |

* Mugford & others (1994)

**Table 5. *Hi-maize*™ addition to pasta.**

| | | *Hi-maize*™ replacement of semolina | | |
|---|---|---|---|---|
| | | 0% | 12% | 24% |
| Moisture content | (%) | 11.6 | 12.7 | 12.3 |
| Dietary fibre* | (%) | 3.3 | 5.6 | 7.9 |
| Ash * | (%) | 0.90 | 0.80 | 0.72 |
| Protein (N×5.7)* | (%) | 14.7 | 13.2 | 11.6 |

* dry solids basis

are sensitive.

*Hi-maize*™ provides the food manufacturer with an opportunity to increase invisibly the dietary fibre content of foods such as pasta (Table 5) and noodles while avoiding the grainy or discoloured appearance that results from the inclusion of traditional sources of dietary fibre.

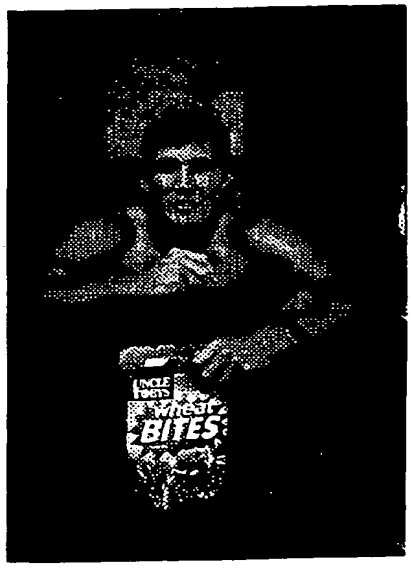
*Hi-maize*™ has been successfully used in products where the dietary fibre content of the food is not a prerequisite. As a partial replacement for flour in ice cream cones, *Hi-maize*™ improves the crispness and keeping qualities of the cone. Alternatively *Hi-maize*™ can be used to reduce the oil pick-up in expanded snacks.

*Hi-maize*™ has potential to be included in pharmaceutical products where those specific health characteristics currently being studied can be used to treat physiological conditions. In this area the definitions of food and pharmaceutical products become blurred.

The development and success of these new consumer products has led to sales of *Hi-maize*™ beyond expectations in the first 18 months since product launch. The implications of this success are already being felt by increased demand for maize cropping, nutritional and analytical research on starch and exports of *Hi-maize*™. Indeed export sales of *Hi-maize*™ already account for 25% of the sales volume and this is expected to rapidly increase with the development of functional foods in Asian markets.

## Conclusion

The current nutritional interest in dietary carbohydrates, such as resistant starch, still offers a number of technical and marketing challenges. These challenges include firstly the need to increase research and popular focus directed on the carbohydrate portion of our diets which has been neglected in the past. Since the 1950s, people have had the misconception that starch is fattening even though its calorific value is only 17 kJ/g as opposed to 37 kJ/g for fat and oils. Secondly, it is important that a reliable and accepted method be developed to determine the resistant starch content in foods. Many methods have been reported but a consensus has yet to be reached in the scientific community as to which in vitro method will best indicate the effect of resistant starch in vivo. Agreement on a method is necessary before the true extent of resistant starch consumption and the full extent of its importance to public health is known. Current methods for determining the dietary fibre content of foods appear to significantly underestimate the levels of resistant starch.




*Wheat Bites* by The Uncle Tobys Co.

*Hi-maize*™ is the result of basic cereal research conducted in Australia and commercialised by Starch Australasia Ltd. The importance of *Hi-maize*™ for product innovation has been accepted by food companies in Australia and overseas and has resulted in significant sales of *Hi-maize*™ and the successful launch of novel consumer products. The nutritional significance of *Hi-maize*™ is growing with the publication of research data. *Hi-maize*™ represents the commitment of Starch Australasia Ltd to ingredient research and product innovation, responding to consumer needs and seeking to improve public health.

I would like to thank AIFST for the honour, recognition and encouragement that it has accorded to Starch Australasia Ltd and my colleagues with the presentation of the Food Industry Innovation Award.

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# Determination of Insoluble, Soluble, and Total Dietary Fiber in Foods and Food Products: Interlaboratory Study

EXHIBIT C

LEON PROSKY
*Food and Drug Administration, Division of Nutrition, Washington, DC 20204*
NILS-GEORG ASP
*University of Lund, Department of Food Chemistry, S-22077 Lund, Sweden*
THOMAS F. SCHWEIZER
*Nestlé Research Centre, Nestec Ltd, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland*
JONATHAN W. DeVRIES and IVAN FURDA
*General Mills, Minneapolis, MN 55427*

Collaborators: F. Alstin; N.-G. Asp; M. Bergstrom-Nielsen; P. A. Burdaspal; C. E. Daugherty; K. Felip; D. Gamblin; T. Geselle; D. T. Gordon; K. Kiekhoefer; S. Reimann; T. F. Schweizer; W. Seibel; G. E. Sipes; A. Sørensen; A. W. Wight; J. W. Zurawski (R. Albert and A. Wilson, *Statistical Consultants*)

**A collaborative study was conducted to determine the insoluble dietary fiber (IDF), soluble dietary fiber (SDF), and total dietary fiber (TDF) content of food and food products by using a combination of enzymatic and gravimetric procedures. The method was basically the same as that developed for TDF only, which was adopted official final action by AOAC, except for changing the concentration of buffer and base and substituting hydrochloric acid for phosphoric acid. These changes were made to improve the robustness of the method. Duplicate blind samples of soy isolate, white wheat flour, rye bread, potatoes, rice, corn bran, oats, Fabulous Fiber, wheat bran, and a high fiber cereal were analyzed by 13 collaborators. Dietary fiber values (IDF, SDF, and TDF) were calculated as the weight of residue minus the weight of protein and ash. The coefficients of variation (CVs) of both the independent TDF determination and the sum of IDF and SDF were better than 15 and 18%, respectively, with the exception of rice and soy isolate. These 2 foods, however, contained only about 1% TDF. The CVs of the IDF were equally good, except for Fabulous Fiber, for which filtration problems occurred. The CVs for the SDF were somewhat high, but these products had very low SDF content. There was excellent agreement between the TDF determined independently and the TDF determined by summing the IDF and SDF. The method for separate determination of IDF and SDF requires further study. The modifications (changes in concentration of buffer and base and the use of hydrochloric acid instead of phosphoric acid) to the official final action method for TDF have been adopted.**

The determination of total dietary fiber (TDF) by an enzymatic-gravimetric procedure has been adopted official final action by AOAC (1). The collaborative study reported here incorporates into this method changes in the concentration of the phosphate buffer, from 0.05 to 0.08M; changes in the concentration of the sodium hydroxide, from 0.171 to 0.275N; and the use of hydrochloric acid instead of phosphoric acid. These changes were introduced to increase the buffering capacity of the incubation mixture without increasing the final phosphate concentration. Considering that insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) often exhibit distinct physiological effects (2), the basic method has been extended to give separate values for SDF and IDF, if desired, as well as TDF (3). The study was designed according to the rules of Youden and Steiner (4).

Received March 23, 1988. Accepted April 14, 1988.
Presented at the 101st AOAC Annual International Meeting, Sept. 14–17, 1987, San Francisco, CA.
The recommendation of the Associate Referee, L. Prosky, to modify the official final action method for determination of total dietary fiber was approved by the Committee on Foods II. Association actions will be published in the report of the Committee, *J. Assoc. Off. Anal. Chem.* (1989) 72, January–February issue.

## Collaborative Study

The 13 collaborators participating in this study were analysts in food companies, universities, and commercial and government laboratories representing 7 countries. Collaborators were sent 10 duplicate blind samples for analysis: (a) soy protein isolate, Supro 610K, Lot No. C3A BK-008, donated by Ralston Purina Co., St. Louis, MO; (b) white wheat flour, low extraction (0.45% ash, 12% protein), donated by General Mills, Inc., Minneapolis, MN; (c) rye bread, Deli-Rye (Giant Foods, Inc., Washington, DC), dried for 4 h at 80°C; (d) potatoes, instant (Giant Foods); (e) rice, enriched long grain (Giant Foods); (f) corn bran, Lot No. CFL 2601A, donated by A. E. Staley Manufacturing Co., Decatur, IL; (g) oats, quick cooking, donated by Quaker Oats Co., Barrington, IL; (h) Fabulous Fiber, a mixture of malto dextrin, whey, psyllium hulls, guar gum, pectins, vitamins, and minerals (Lewis Laboratories International, Ltd, Westport, CT); (i) wheat bran, AACC certified food grade, purchased from AACC, St. Paul, MN; (j) high fiber cereal AL T., donated by Farma Food, Inc., Washington, DC.

The rye bread, potatoes, rice, oats, wheat bran, and high fiber cereal were ground to a uniform size of 350 μm in a Microjet 10 centrifugal mill (Quartz Technology, Inc., Westbury, NY). No heating of the products occurred during this procedure.

Test samples were placed in plastic bags with a sample letter taped to each bag. None of the test samples had >10% fat; therefore, fat extraction was not recommended. Each test sample was to be dried at 70°C in a vacuum oven (preferred method), or overnight in a 105°C air oven, and stored in a desiccator until analyzed.

The collaborators were further instructed to weigh test portions to the nearest 0.1 mg and express % IDF, SDF, and TDF to 2 decimal places on the data sheets provided. The formulas for the calculation of % IDF, SDF, and TDF were also provided to each collaborator.

The collaborators were to provide the 3 enzymes, Termamyl, amyloglucosidase, and protease, needed to carry out the study.

## METHOD

### *Principle*

Duplicate test portions of dried foods, fat-extracted if they contain >10% fat, are gelatinized with Termamyl (heat-stable α-amylase), and then enzymatically digested with protease and amyloglucosidase to remove protein and starch.

*Insoluble dietary fiber.*—The residue is filtered and washed with water. The filtrate and wash are saved. The residue (IDF) is washed with 95% ethanol and acetone, dried, and weighed.

One duplicate is analyzed for protein, and the other is incinerated at 525°C for ash. IDF is weight of residue less weight of protein and ash.

*Soluble dietary fiber.*—Four volumes of 95% ethanol are added to the combined filtrate and water washings to precipitate SDF. The precipitate is filtered and washed with 78% ethanol, 95% ethanol, and acetone, and is then dried and weighed. One duplicate is analyzed for protein, and the other is incinerated at 525°C for ash. SDF is weight of residue less weight of protein and ash.

*Total dietary fiber.*—See references 1 and 5, sec. **43.A14.**

### *Apparatus*

See references 1 and 5, sec. **48.A15.**

### *Reagents*

See references 1 and 5, sec. **43.A16**; substitute the following for *Reagents* **(d)**, **(h)**, and **(i)**.

**(d)** *Phosphate buffer.*—0.08M, pH 6.0. Dissolve 1.400 g Na phosphate dibasic, anhydrous ($Na_2HPO_4$) (or 1.753 g dihydrate) and 9.68 g Na phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$) (or 10.94 g dihydrate) in ca 700 mL water. Dilute to 1 L with water. Check pH with pH meter.

**(h)** *Sodium hydroxide solution.*—0.275N. Dissolve 11.00 g NaOH in ca 700 mL water in 1 L volumetric flask. Dilute to volume with water.

**(i)** *Hydrochloric acid solution.*—0.325N. Dilute stock solution of known titer, e.g., 325 mL 1M HCl, to 1 L with water.

### *Enzyme Purity*

See reference 5, sec. **43.A17.** *Note*: β-Glucan (barley gum) is now available from Sigma Chemical Co. (No. 7391).

### *Sample Preparation*

See references 1 and 5, sec. **43.A18.**

### *Determination*

See reference 5, sec. **43.A19**, except use 0.275N NaOH instead of 0.171N NaOH, 0.325M HCl instead of 0.205M $H_3PO_4$, and 0.08M phosphate buffer instead of 0.05M phosphate buffer.

**(a)** *Insoluble dietary fiber.*—(*1*) Weigh crucible containing Celite to nearest 0.1 mg, then wet and redistribute bed of Celite in crucible by using stream of water from wash bottle. Apply suction to crucible to draw Celite onto fritted glass as even mat. Maintain suction and quantitatively transfer precipitate from enzyme digest through crucible into preweighed suction flask.

(*2*) Wash residue with two 10 mL portions of water. Save filtrate and water washings for determination of SDF (density of filtrate is close to 1).

(*3*) Wash residue with two 10 mL portions of 95% ethanol and then with two 10 mL portions of acetone. With some residues, gum may form, trapping liquid. If so, break surface film with spatula to improve filtration. Long filtration times can be avoided by careful intermittent suction throughout filtration. Normal suction can be applied at washing. Back-bubbling with air, if available, will also speed filtration.

(*4*) Dry crucible containing residue overnight in 70°C vacuum oven or 105°C air oven. Cool in desiccator and weigh crucible, Celite, and residue to nearest 0.1 mg. Subtract crucible and Celite weight to determine weight of residue.

(*5*) Analyze residue from 1 determination of set of duplicates for protein (6). Use nitrogen × 6.25 as conversion factor.

(*6*) Incinerate residue of duplicate determination 5 h at 525°C. Cool in desiccator and weigh crucible to nearest 0.1 mg. Subtract crucible and Celite weight to determine ash.

**(b)** *Soluble dietary fiber.*—(*1*) Adjust weight of combined filtrate and water washings from IDF procedure to 100 g with water.

(*2*) Transfer solution to beaker or Erlenmeyer flask. Add four 100 mL portions of 95% ethanol preheated to 60°C. Rinse suction flask with some of ethanol.

(*3*) Allow precipitate to form at room temperature for 60 min.

(*4*) Weigh crucible containing Celite to nearest 0.1 mg, then wet and redistribute bed of Celite in crucible using stream of 78% ethanol from wash bottle. Apply suction to crucible to draw Celite onto fritted glass as even mat. When fiber is filtered, Celite separates fiber from fritted glass of crucible, allowing for easy removal of crucible contents.

(*5*) Filter enzyme digest through crucible. Wash residue successively with three 20 mL portions of 78% ethanol, two 10 mL portions of 95% ethanol, and two 10 mL portions of acetone. With some residues, gum may form, trapping liquid. If so, break surface film with spatula to improve filtration. Long filtration times can be avoided by careful intermittent suction throughout filtration. Normal suction can be applied at washing. Back-bubbling with air, if available, will also speed filtration.

(*6*) Proceed as in determination of IDF, steps (*4*) through (*6*).

**(c)** *Total dietary fiber.*—Adjust volume of enzyme digest to 100 mL with water and proceed to step (*2*) of determination of SDF. Alternatively, add four 100 mL portions of 95% ethanol preheated to 60°C directly to known volume of enzyme digest and proceed to step (*3*) of determination of SDF.

### Calculations

Calculations have been simplified by use of new data sheets and equations. Figure 1 shows the new blank and test sample data sheets and equations for calculation of dietary fiber, which have rectified the problems associated with the calculations.

## Results and Discussion

The determination of TDF by an enzymatic–gravimetric procedure was adopted official final action by AOAC in March 1986 (1). The collaborative study undertaken and reported in this paper uses the same 3 enzymes; however, the concentration of phosphate buffer has been increased from 0.05 to 0.08M, the concentration of sodium hydroxide has been increased from 0.171 to 0.275N, and a hydrochloric acid solution has been substituted for the phosphoric acid solution. The changes were introduced into the adopted method to improve its robustness. The increased buffering capacity of the initial incubation mixture avoids pH adjustments when acidic products (e.g., fruit pulps) are analyzed. The change in the acid used became necessary to avoid a concomitant increase in the final phosphate concentration, which had given rise to salt coprecipitation (7). We also hoped to establish methods for SDF and IDF, in addition to TDF, by filtering the IDF before precipitating the SDF with ethanol. We sought to determine the agreement between the independent method for TDF compared with TDF derived by summing SDF and IDF. A collaborative study in Switzerland (8) gave excellent results for TDF using the AOAC method with the above changes included.

**AOAC DIETARY FIBER METHOD BLANK DATA SHEET**

BLANKS

| | a | | b | | c | | d | |
|---|---|---|---|---|---|---|---|---|
| Crucible + Celite + tare Weight (mg) | | | | | | | | |
| Crucible + Celite + Residue Weight (mg) | | | | | | | | |
| Residue Weight (mg) | $R_1$ | $R_2$ | $R_1$ | $R_2$ | $R_1$ | $R_2$ | $R_1$ | $R_2$ |
| Protein (mg) P | | | | | | | | |
| Crucible + Celite + Ash weight (mg) | | | | | | | | |
| Ash weight (mg) A | | | | | | | | |
| Blanks (mg) | | | | | | | | |
| Mean Blank (a + b + c + d)/4 (mg) B | | | | | | | | |

$$\text{Blanks (mg)} = \frac{R_1 + R_2}{2} - P - A$$

**AOAC DIETARY FIBER METHOD SAMPLE DATA SHEET**

| | Sample | | | | Sample | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 1 | | 2 | |
| 1. Sample Weight (mg) | $m_1$ | $m_2$ | $m_1$ | $m_2$ | $m_1$ | $m_2$ | $m_1$ | $m_2$ |
| 2. Crucible + Celite tare weight (mg) | | | | | | | | |
| 3. Crucible + Celite + Residue weight (mg) | | | | | | | | |
| 4. Residue Weight (mg) | $R_1$ | $R_2$ | $R_1$ | $R_2$ | $R_1$ | $R_2$ | $R_1$ | $R_2$ |
| 5. Protein (mg) P | | | | | | | | |
| 6. Crucible + Celite + Ash weight (mg) | | | | | | | | |
| 7. Ash Weight (mg) A | | | | | | | | |
| 8. Mean Blank (mg) B | | | | | | | | |
| 9. Dietary Fiber (%) | | | | | | | | |

$$\text{Dietary Fiber (\%)} = \frac{\frac{R_1 + R_2}{2} - P - A - B}{\frac{m_1 + m_2}{2}} \times 100$$

**Figure 1. Sample and blank data sheet and equations for calculation of blank and percent dietary fiber.**

The results of the individual analyses for IDF, SDF, TDF (the sum of IDF and SDF), and TDF by independent analysis are shown in Table 1. The results from Collaborator 3 were not included because the analyst did not use the proper enzymes. Collaborator 4 did not report any IDF and SDF results for soy isolate and white wheat flour. Collaborator 10 failed to report results in time for this report. Collaborators 1 and 11 did not report any values for the independent determination of TDF.

In the case of white wheat flour, one laboratory reported a value that was an outlier by both the Grubbs and Dixon tests for SDF and another laboratory reported a Grubbs outlier for TDF. For potatoes, one laboratory had a Cochran outlier for SDF; for oats, one laboratory had a Grubbs outlier, and another had a result that was an outlier by both the Cochran and Grubbs tests for IDF determination. Fabulous Fiber presented a different problem in that many of the collaborators said they could not filter the fiber, so they deviated from the procedure by centrifuging the samples. Others did not report values because of the filtration problem. Four of the collaborators did not experience any problems with determining SDF and IDF and 7 did not experience any problems with the independent TDF determination. All values were used in the final statistics for soy isolate, rye bread, rice, corn bran, wheat bran, and high fiber cereal.

The measures of precision for IDF, SDF, and TDF are shown in Tables 2–4. In these tables, RSD, is the repeatability relative standard deviation and is a measure (in percent) of how well a typical laboratory can consistently obtain the same results under the same conditions. In other words, RSD, measures within-laboratory variability. $RSD_R$ is the reproducibility relative standard deviation and is a measure of among-laboratory variability. The Fabulous Fiber sample had a reproducibility coefficient of 49% for IDF (Table 2); however, this was based on data from only 4 laboratories. This was mainly due to the filtration problems associated with this partly soluble, viscous, but thixotropic sample. The high coefficients of reproducibility for rice and soy isolate were due mainly to the low IDF in these products. The fact that the IDF for soy isolate was higher than the TDF mea-

**Table 1. Collaborative results (blind duplicates) of determination of IDF, SDF, and TDF by enzymatic–gravimetric method**

| Coll.[a] | Insoluble dietary fiber, % | Soluble dietary fiber, % | Total dietary fiber: Sum of insoluble + soluble fiber, % | Total dietary fiber: Independent analysis, % |
|---|---|---|---|---|
| | | Corn bran | | |
| 1 | 86.98 | 0.28 | 87.26 | —[b] |
| | 86.82 | 0.47 | 87.29 | — |
| 2 | 88.26 | 0.28 | 88.54 | 88.02 |
| | 87.95 | 0.56 | 88.51 | 87.56 |
| 4 | 88.22 | 0.80 | 89.02 | 86.57 |
| | 88.67 | 0.85 | 89.52 | 87.70 |
| 5 | 88.63 | 0.00 | 88.63 | 88.70 |
| | 87.79 | 0.01 | 87.80 | 87.98 |
| 6 | 87.97 | 0.51 | 88.48 | 87.88 |
| | 87.25 | 0.50 | 87.75 | 88.11 |
| 7 | 86.51 | 0.52 | 87.03 | 87.64 |
| | 86.28 | 0.97 | 87.25 | 87.78 |
| 8 | 87.17 | 0.00 | 87.17 | 84.90 |
| | 86.74 | 0.00 | 86.74 | 83.67 |
| 11 | 88.30 | 0.20 | 88.50 | —[b] |
| | 88.00 | 0.00 | 88.00 | — |
| 12 | 87.63 | 0.63 | 88.26 | 87.97 |
| | 88.09 | 0.44 | 88.53 | 87.91 |
| 13 | 86.58 | 0.68 | 87.26 | 87.23 |
| | 86.97 | 0.59 | 87.56 | 87.92 |
| 14 | 86.55 | 0.40 | 86.95 | 85.25 |
| | 86.96 | 0.003 | 86.96 | 85.46 |
| | | Fabulous Fiber | | |
| 1 | —[c] | —[c] | — | —[b] |
| | —[c] | —[c] | — | — |
| 2 | 5.87[d] | 13.52 | 19.39 | 17.05 |
| | 6.09[d] | 13.18 | 19.27 | 17.66 |
| 5 | 9.28 | 5.95 | 15.23 | 18.65 |
| | 7.71 | 6.56 | 14.27 | 17.09 |
| 6 | 7.39[d] | 10.83 | 18.22 | 19.75 |
| | 11.47[d] | 8.61 | 20.08 | 19.85 |
| 7 | 5.40 | 12.10 | 17.50 | 17.12 |
| | 4.04 | 12.42 | 16.46 | 16.86 |
| 8 | 6.85 | 9.37 | 16.22 | 16.53 |
| | 7.58 | 9.70 | 17.28 | 15.48 |
| 11 | 14.60 | 2.20 | 16.80 | —[b] |
| | 15.60 | 3.00 | 18.60 | — |
| 12 | —[c] | —[c] | — | 13.75 |
| | —[c] | —[c] | — | 14.21 |
| 13 | —[c] | —[c] | — | —[e] |
| | —[c] | —[c] | — | —[e] |
| 14 | —[c] | —[c] | — | 16.41 |
| | —[c] | —[c] | — | 15.19 |

**Table 1. Continued**

| Coll.[a] | Insoluble dietary fiber, % | Soluble dietary fiber, % | Total dietary fiber: Sum of insoluble + soluble fiber, % | Total dietary fiber: Independent analysis, % |
|---|---|---|---|---|
| | | High fiber cereal | | |
| 1 | 29.70 | 2.19 | 31.89 | —[b] |
| | 29.95 | 1.36 | 31.31 | — |
| 2 | 29.97 | 1.84 | 31.81 | 29.64 |
| | 32.04 | 1.70 | 33.74 | 32.24 |
| 4 | 32.80 | 2.63 | 35.43 | 33.79 |
| | 31.65 | 2.49 | 34.14 | 34.41 |
| 5 | 28.04 | 1.16 | 29.20 | 33.53 |
| | 30.88 | 1.52 | 32.40 | 34.02 |
| 6 | 30.40 | 1.15 | 31.55 | 31.82 |
| | 30.20 | 2.26 | 32.46 | 32.95 |
| 7 | 29.08 | 1.89 | 30.97 | 32.28 |
| | 29.74 | 1.69 | 31.43 | 31.52 |
| 8 | 29.57 | 1.22 | 30.79 | 31.70 |
| | 30.50 | 0.75 | 31.25 | 29.97 |
| 11 | 30.20 | 3.40 | 33.60 | —[b] |
| | 31.40 | 1.40 | 32.80 | — |
| 12 | 32.14 | 2.66 | 34.80 | 31.93 |
| | 28.35 | 2.39 | 30.74 | 32.69 |
| 13 | 28.22 | 2.32 | 30.54 | 31.47 |
| | 29.61 | 1.96 | 31.57 | 31.52 |
| 14 | 28.44 | 2.40 | 30.84 | 31.81 |
| | 30.23 | 1.07 | 31.30 | 31.97 |
| | | Oats | | |
| 1 | 5.38 | 3.68 | 9.06 | —[b] |
| | 6.66 | 3.57 | 10.23 | — |
| 2 | 5.96 | 3.47 | 9.43 | 11.37 |
| | 7.25 | 3.71 | 10.96 | 12.73 |
| 4 | 5.71 | 6.20 | 11.91 | 11.58 |
| | 5.36 | 5.69 | 11.05 | 11.04 |
| 5 | 5.79[d,f] | 2.66 | 8.45 | 14.81 |
| | 10.60 | 2.51 | 13.11 | 14.15 |
| 6 | 10.61[f,g] | 3.85 | 14.46 | 16.12 |
| | 8.98 | 4.68 | 13.66 | 15.01 |
| 7 | 5.00 | 3.95 | 8.95 | 11.44 |
| | 5.34 | 4.09 | 9.43 | 10.78 |
| 8 | 5.51 | 3.31 | 8.82 | 11.26 |
| | 6.18 | 3.43 | 9.61 | 10.11 |
| 11 | 7.10 | 4.60 | 11.70 | —[b] |
| | 5.40 | 3.30 | 8.70 | — |
| 12 | 5.60 | 5.96 | 11.56 | 11.36 |
| | 5.40 | 5.97 | 11.37 | 10.75 |
| 13 | 4.99 | 3.54 | 8.53 | 9.92 |
| | 4.85 | 3.90 | 8.75 | 8.22 |
| 14 | 5.35 | 4.63 | 9.98 | 10.20 |
| | 5.92 | 5.91 | 11.83 | 11.24 |

sured independently suggests that, when the sample was prepared for IDF determination, another substance may have been precipitated in addition to fiber. The other foods gave reasonable results, with the reproducibility proportional to the IDF content of the product.

Table 3 shows the measures of precision for the determination of SDF. The reproducibility coefficients were very high for rice and soy isolate (128 and 100%, respectively) and in the 20–50% range for the remaining foods except corn bran ($RSD_R$ 79%). The high variability of reproducibility is due mainly to the very low SDF content of the foods, the exception being Fabulous Fiber, which had about 9% SDF and a 45% coefficient of reproducibility. The problem with filtration of this product has been discussed earlier. It is nonetheless noteworthy that the variable recoveries in either IDF or SDF compensated for each other, and the sums were confined to a narrower range. Thus, for Fabulous Fiber, the $RSD_R$ of the sum would be only 8%, compared with over 40% of the individual fractions.

Table 4 shows the TDF independent analysis values and the measures of precision. All foods analyzed had <15% coefficient of reproducibility with the exception of rice and soy isolate, which had $RSD_R$ values of 70 and 88%, respectively. These 2 foods have always had large $RSD_R$ values because of their low fiber content (5, 7). While $RSD_r$ and $RSD_R$ for TDF in the present study are not quite as good as

**Table 1. Continued**

| Coll.[a] | Insoluble dietary fiber, % | Soluble dietary fiber, % | Total dietary fiber: Sum of insoluble + soluble fiber, % | Total dietary fiber: Independent analysis, % |
|---|---|---|---|---|
| | | Potatoes | | |
| 1 | 4.97 | 2.12 | 7.09 | —[b] |
| | 3.98 | 2.46 | 6.44 | — |
| 2 | 5.01 | 2.29 | 7.30 | 6.79 |
| | 4.65 | 2.34 | 6.99 | 6.66 |
| 4 | 5.37 | 2.56 | 7.93 | 7.04 |
| | 4.81 | 3.26 | 8.07 | 8.19 |
| 5 | 5.65 | 1.57 | 7.22 | 7.69 |
| | 5.59 | 1.37 | 7.96 | 7.80 |
| 6 | 4.60 | 1.98 | 6.58 | 6.60 |
| | 4.84 | 2.19 | 7.03 | 7.19 |
| 7 | 5.16 | 1.70 | 6.86 | 6.98 |
| | 5.09 | 1.76 | 6.85 | 7.22 |
| 8 | 4.93 | 1.35 | 6.28 | 7.17 |
| | 5.67 | 1.26 | 6.93 | 4.97 |
| 11 | 4.60 | 3.50[a,b] | 8.10 | —[b] |
| | 5.30 | 1.80 | 7.10 | — |
| 12 | 5.12 | 1.62 | 6.74 | 6.90 |
| | 4.81 | 1.84 | 6.65 | 7.15 |
| 13 | 3.65 | 2.38 | 6.03 | 7.11 |
| | 3.52 | 2.70 | 6.22 | 6.62 |
| 14 | 4.55 | 2.58 | 7.13 | 4.90 |
| | 4.84 | 2.51 | 7.35 | 5.04 |
| | | Rice | | |
| 1 | 0.45 | 0.49 | 0.94 | —[b] |
| | 0.63 | 0.00 | 0.63 | — |
| 2 | 0.62 | 0.06 | 0.68 | 1.03 |
| | 0.81 | 0.11 | 0.92 | 0.78 |
| 4 | 1.02 | 0.00 | 1.02 | 2.08 |
| | 1.13 | 0.36 | 1.49 | 1.04 |
| 5 | 0.85 | 0.17 | 1.02 | 2.31 |
| | 0.86 | 0.01 | 0.87 | 0.97 |
| 6 | 0.92 | 0.00 | 0.92 | 1.05 |
| | 0.73 | 0.09 | 0.82 | 1.11 |
| 7 | 1.11 | 0.52 | 1.63 | 1.43 |
| | 1.08 | 0.53 | 1.61 | 1.36 |
| 8 | 0.67 | 0.13 | 0.80 | 0.70 |
| | 1.49 | 0.00 | 1.49 | 1.15 |
| 11 | 0.60 | 0.00 | 0.60 | —[b] |
| | 1.00 | 0.00 | 1.00 | — |
| 12 | 0.55 | 0.00 | 0.55 | 0.76 |
| | 0.59 | 0.02 | 0.61 | 0.52 |
| 13 | 0.16 | 0.00 | 0.16 | 0.58 |
| | 0.34 | 0.18 | 0.52 | 0.48 |
| 14 | 0.56 | 0.20 | 0.76 | 0.00 |
| | 0.41 | 0.42 | 0.83 | 0.00 |

**Table 1. Continued**

| Coll.[a] | Insoluble dietary fiber, % | Soluble dietary fiber, % | Total dietary fiber: Sum of insoluble + soluble fiber, % | Total dietary fiber: Independent analysis, % |
|---|---|---|---|---|
| | | Rye bread | | |
| 1 | 5.47 | 1.75 | 7.22 | —[b] |
| | 6.21 | 1.81 | 8.02 | — |
| 2 | 5.17 | 1.75 | 6.92 | 6.73 |
| | 5.50 | 1.86 | 7.36 | 6.99 |
| 4 | 5.08 | 2.23 | 7.31 | 6.97 |
| | 4.75 | 2.20 | 6.95 | 7.08 |
| 5 | 6.41 | 1.00 | 7.41 | 7.21 |
| | 6.91 | 1.10 | 8.01 | 7.19 |
| 6 | 5.27 | 0.64 | 5.91 | 6.94 |
| | 5.09 | 1.42 | 6.51 | 7.25 |
| 7 | 5.18 | 1.35 | 6.53 | 6.26 |
| | 5.15 | 1.29 | 6.44 | 6.36 |
| 8 | 5.41 | 1.60 | 7.01 | 6.56 |
| | 6.28 | 1.30 | 7.58 | 4.68 |
| 11 | 5.90 | 0.90 | 6.80 | —[b] |
| | 6.40 | 0.90 | 7.30 | — |
| 12 | 6.16 | 1.74 | 7.90 | 6.88 |
| | 5.83 | 1.70 | 7.53 | 6.69 |
| 13 | 3.72 | 1.88 | 5.60 | 5.94 |
| | 3.64 | 1.69 | 5.33 | 5.98 |
| 14 | 4.39 | 2.03 | 6.42 | 8.80 |
| | 5.22 | 1.66 | 6.88 | 6.29 |
| | | Soy isolate | | |
| 1 | 9.32 | 0.00 | 9.32 | —[b] |
| | 7.00 | 0.00 | 7.00 | — |
| 2 | 6.69 | 0.30 | 6.99 | 0.79 |
| | 7.24 | 0.23 | 7.47 | 0.55 |
| 4 | —[c] | —[c] | — | 0.00 |
| | — | — | — | 0.00 |
| 5 | 6.63 | 0.07 | 6.70 | 1.10 |
| | 6.79 | 0.18 | 6.97 | 0.49 |
| 6 | 6.56 | 0.70 | 7.26 | 0.06 |
| | 4.86 | 1.04 | 5.90 | 1.65 |
| 7 | 2.84 | 1.30 | 4.14 | 3.76 |
| | 2.52 | 0.60 | 3.12 | 4.44 |
| 8 | 7.73 | 1.37 | 9.10 | 2.95 |
| | 8.28 | 0.59 | 8.87 | 1.54 |
| 11 | 7.80 | 0.00 | 7.80 | —[b] |
| | 7.80 | 0.00 | 7.80 | — |
| 12 | 5.55 | 0.35 | 5.90 | 1.17 |
| | 7.53 | 0.00 | 7.53 | 1.10 |
| 13 | 5.69 | 0.68 | 6.37 | 2.06 |
| | 4.72 | 0.85 | 5.57 | 0.58 |
| 14 | 4.21 | 0.88 | 5.09 | 2.81 |
| | 6.39 | 0.00 | 6.39 | 3.18 |

those obtained in the earlier collaborative study (5), they are still acceptable and may be due to the much heavier workload involved in the present study, in which 3 times as many crucibles were handled as in the earlier study. For the 8 products already collaboratively studied, the measures of precision have been calculated after elimination of only 2 results of 142, whereas in the previous study, 6 of 160 results were eliminated. The average TDF values were nearly identical to those from an earlier study (7). Thus, the changes in the buffer concentrations and the acid did not alter the performance of the method.

When the TDF by independent analysis is compared with the TDF obtained by summing IDF and SDF, the results are remarkably similar. With the exception of soy isolate, which had 4 times as much IDF as TDF (due to precipitation of some material other than fiber in the fractionation process) and therefore about 4 times as much TDF calculated by summing IDF and SDF as compared with the independent determination of TDF, all other values for TDF obtained by summing IDF and SDF were acceptable. This would eliminate the necessity of determining TDF independently except when TDF alone is desired. However, the precision of the method for the individual fractions, especially for SDF, is not yet satisfactory. This is due in part to choosing products with very low SDF or with special problems. Further studies of fruits, vegetables, and leguminous seeds are needed.

**Table 1. Continued**

| Coll.[a] | Insoluble dietary fiber, % | Soluble dietary fiber, % | Total dietary fiber: Sum of insoluble + soluble fiber, % | Total dietary fiber: Independent analysis, % |
|---|---|---|---|---|
| | | Wheat bran | | |
| 1 | 40.98 | 2.78 | 43.76 | —[b] |
| | 40.88 | 2.75 | 43.63 | — |
| 2 | 42.33 | 2.91 | 45.24 | 44.54 |
| | 39.64 | 2.31 | 41.95 | 41.97 |
| 4 | 41.28 | 3.43 | 44.71 | 43.02 |
| | 40.77 | 4.27 | 45.04 | 42.66 |
| 5 | 42.08 | 1.90 | 43.98 | 45.73 |
| | 43.72 | 1.59 | 45.31 | 46.31 |
| 6 | 42.40 | 3.11 | 45.51 | 45.04 |
| | 41.23 | 2.94 | 44.17 | 42.37 |
| 7 | 40.61 | 2.81 | 43.42 | 43.27 |
| | 41.51 | 2.54 | 44.05 | 43.78 |
| 8 | 39.91 | 3.13 | 43.04 | 43.18 |
| | 39.91 | 2.95 | 42.86 | 43.05 |
| 11 | 44.10 | 2.90 | 47.00 | —[b] |
| | 43.80 | 2.40 | 46.20 | — |
| 12 | 44.05 | 3.31 | 47.36 | 44.78 |
| | 42.76 | 3.23 | 45.99 | 44.66 |
| 13 | 40.24 | 3.06 | 43.30 | 44.32 |
| | 40.46 | 2.75 | 43.21 | 44.03 |
| 14 | 40.69 | 3.49 | 44.18 | 42.90 |
| | 41.52 | 2.51 | 44.03 | 44.03 |
| | | White wheat flour | | |
| 1 | 1.31 | 1.05 | 2.36 | —[b] |
| | 1.48 | 1.00 | 2.48 | — |
| 2 | 1.62 | 1.14 | 2.76 | 3.19 |
| | 1.60 | 1.46 | 3.06 | 2.92 |
| 5 | 1.83 | 1.10 | 2.93 | 3.67 |
| | 1.98 | 0.90 | 2.88 | 2.60 |
| 6 | 1.55 | 1.10 | 2.65 | 4.08 |
| | 1.76 | 1.77 | 3.53 | 3.11 |
| 7 | 1.90 | 0.74 | 2.64 | 3.16 |
| | 1.67 | 1.36 | 3.03 | 3.39 |
| 8 | 1.80 | 1.16 | 2.96 | 3.40 |
| | 1.88 | 1.14 | 3.02 | 2.88 |
| 11 | 1.60 | 0.10[d] | 1.70 | —[b] |
| | 1.80 | 0.30 | 2.10 | — |
| 12 | 1.43 | 1.26 | 2.69 | 3.18 |
| | 1.30 | 0.95 | 2.25 | 2.98 |
| 13 | 0.64 | 1.26 | 1.90 | 2.67 |
| | 1.14 | 1.20 | 2.34 | 2.58 |
| 14 | 1.19 | 0.99 | 2.18 | 1.56[d,g] |
| | 1.62 | 1.53 | 3.15 | 1.10 |

[a] The number 9 was not assigned. Results from Collaborator 3 are not included; enzymes used in analysis were not those specified. Collaborator 10 did not report results in time for evaluation.
[b] Collaborators 1 and 11 did not report results for independent analysis of TDF.
[c] Collaborator experienced problem with filtration; no results reported.
[d] Collaborator reported filtration problems.
[e] Cochran and Grubbs outlier.
[f] Outlier designation refers to both samples of the pair.
[g] Grubbs outlier.
[h] Cochran outlier.
[i] No results reported.
[j] Grubbs and Dixon outlier.

### *Recommendation*

It is recommended that the modifications to the official final action method for total dietary fiber (**43.A14-43.A20**) be adopted.

**Table 2. Measures of precision for determining IDF**

| Product | Average IDF, % | No. of colls | Repeatability $RSD_r$, % | Reproducibility $RSD_R$, % |
|---|---|---|---|---|
| Corn bran | 87.47 | 11 | 0.38 | 0.87 |
| Fabulous Fiber | 9.13 | 4 | 7.31 | 48.79 |
| High fiber cereal | 30.14 | 11 | 4.24 | 4.33 |
| Oats | 5.66 | 9 | 11.11 | 12.07 |
| Potatoes | 4.85 | 11 | 7.25 | 11.94 |
| Rice | 0.75 | 11 | 28.38 | 41.52 |
| Rye bread | 5.42 | 11 | 6.66 | 15.67 |
| Soy isolate | 6.31 | 10 | 15.29 | 28.56 |
| Wheat bran | 41.59 | 11 | 1.98 | 3.40 |
| White wheat flour | 1.56 | 10 | 11.57 | 21.03 |

**Table 3. Measures of precision for determining SDF**

| Product | Average SDF, % | No. of colls | Repeatability $RSD_r$, % | Reproducibility $RSD_R$, % |
|---|---|---|---|---|
| Corn bran | 0.40 | 11 | 40.22 | 78.57 |
| Fabulous Fiber | 8.95 | 6 | 8.07 | 44.72 |
| High fiber cereal | 1.88 | 11 | 32.64 | 34.74 |
| Oats | 4.21 | 11 | 10.78 | 26.73 |
| Potatoes | 2.14 | 11 | 19.27 | 27.88 |
| Rice | 0.19 | 11 | 100.03 | 127.52 |
| Rye bread | 1.54 | 11 | 13.19 | 28.67 |
| Soy isolate | 0.46 | 10 | 71.86 | 100.85 |
| Wheat bran | 2.87 | 11 | 12.01 | 19.78 |
| White wheat flour | 1.17 | 9 | 23.52 | 23.52 |

**Table 4. Measures of precision for determining TDF independently**

| Product | Average TDF, % | No. of colls | Repeatability $RSD_r$, % | Reproducibility $RSD_R$, % |
|---|---|---|---|---|
| Corn bran | 87.13 | 9 | 0.55 | 1.62 |
| Fabulous Fiber | 16.83 | 7 | 3.79 | 11.19 |
| High fiber cereal | 32.18 | 9 | 2.63 | 3.98 |
| Oats | 11.31 | 8 | 6.51 | 14.32 |
| Potatoes | 6.78 | 9 | 9.15 | 13.93 |
| Rice | 1.01 | 9 | 54.32 | 70.42 |
| Rye bread | 6.61 | 9 | 11.14 | 12.20 |
| Soy isolate | 1.57 | 9 | 41.81 | 88.02 |
| Wheat bran | 43.87 | 9 | 2.14 | 2.70 |
| White wheat flour | 3.09 | 7 | 13.58 | 13.58 |


## Acknowledgments

The Associate Referee expresses appreciation to the following collaborators who participated in the study:

F. Alstin, Tecator Laboratories, Hoganas, Sweden

N.-G. Asp, University of Lund, Lund, Sweden

M. Bergstrom-Nielsen and A. Sørensen, National Food Agency, Søborg, Denmark

P. A. Burdaspal, Centro Nacional De Alimentacion y Nutricion, Madrid, Spain

C. E. Daugherty and K. Felip, Campbell Institute for Research and Technology, Camden, NJ

D. Gamblin, Ralston Purina Co., St. Louis, MO

T. Geselle, Kellogg Co., Battle Creek, MI

D. T. Gordon, University of Missouri, Columbia, MO

K. Kiekhoefer and J. W. Zurawski, General Mills, Inc., Minneapolis, MN

T. F. Schweizer and S. Reimann, Nestec Ltd, Vevey, Switzerland

W. Seibel, Federal Research Center for Grain and Potato Processing, Detmold, FGR

G. E. Sipes, The Quaker Oats Co., Barrington, IL

A. W. Wight, National Food Research Institute, Pretoria, South Africa

Appreciation is also extended to Richard Albert and Anne Wilson, Food and Drug Administration, who conducted the statistical evaluation of the data.

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SPECIAL GUEST EDITOR SECTION

# Measurement of Novel Dietary Fibers

BARRY V. McCLEARY and PATRICIA ROSSITER
Megazyme International Ireland Ltd., Bray Business Park, Southern Cross Rd, Bray, County Wicklow, Ireland

**With the recognition that resistant starch (RS) and nondigestible oligosaccharides (NDO) act physiologically as dietary fiber (DF), a need has developed for specific and reliable assay procedures for these components. The ability of AOAC DF methods to accurately measure RS is dependent on the nature of the RS being analyzed. In general, NDO are not measured at all by AOAC DF Methods 985.29 or 991.43, the one exception being the high molecular weight fraction of fructo-oligosaccharides. Values obtained for RS, in general, are not in good agreement with values obtained by in vitro procedures that more closely imitate the in vivo situation in the human digestive tract. Consequently, specific methods for the accurate measurement of RS and NDO have been developed and validated through interlaboratory studies. In this paper, modifications to AOAC fructan Method 999.03 to allow accurate measurement of enzymically produced fructo-oligosaccharides are described. Suggested modifications to AOAC DF methods to ensure complete removal of fructan and RS, and to simplify pH adjustment before amyloglucosidase addition, are also described.**

The area of definition and measurement of dietary fiber (DF) is currently in a state of flux and rapid evolution. Recently updated definitions of DF (1, 2) highlight the importance of physiological considerations, as discussed in detail by Champ et al. (3). As a consequence, resistant starch (RS) and nondigestible oligosaccharides (NDO) are now recognized as DF. This does not mean that the analytical data collected to date are incorrect but, for some sample materials, DF may be underestimated. In a recent report (2), the Food and Nutrition Board of the Institute of Health, under the oversight of the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, erroneously concluded that the current situation regarding labeling and defining DF in the United States and elsewhere was arbitrary. This conclusion was based on their perception that there was too heavy a reliance on analytical methodology rather than on an accurate definition that includes its role in health. In fact, early researchers specifically developed methods to remove materials that are readily and rapidly digested in the small intestine, in line with physiological considerations.

For some years, the "gold standard" method for the measurement of total dietary fiber (TDF) has been AOAC Method 985.29 (the Prosky method; 4, 5). This method has been modified to allow the measurement of soluble and insoluble DF and the incorporation of improved buffers (6). The recognition that RS and NDO also act physiologically as DF has necessitated the development of methods for measurement of these specific components and for parallel modifications to the TDF methods to ensure that all of these components are solubilized/hydrolyzed and that none of these specific components are measured twice.

In the current paper, recent developments in methods for the measurement of fructo-oligosaccharides (FOS) and RS are described in detail. Modifications to the DF methods to ensure complete removal of RS and fructan are described, as well as an overview of methods for the measurement of resistant maltodextrins and galacto-oligosaccharides.

## METHODS

### *Reagents and Standards*

(a) *Enzymes.*—Amyloglucosidase (Cat. No. E-AMGDF), thermostable α-amylase (Cat. No. E-BLAAM), protease (Subtilisin A; Cat. No. E-BSPRT), fructanase enzyme mixture (Cat. No. E-FRMXLQ), Glucose assay kit (Cat. No. K-GLUC), Fructan assay kit (Cat. No. K-FRUC), Fructan HK assay kit (Cat. No. K-FRUCHK), and Resistant Starch assay kit (Cat. No. K-RSTAR) were obtained from Megazyme International Ireland Ltd. (Bray, Ireland).

(b) *Substrates.*—Barley β-glucan (medium viscosity; Cat. No. P-BGBM), pectin (Cat. No. P-CITPN), and wheat arabinoxylan (Cat. No. P-WAXM) were from Megazyme. ACS soluble starch (Cat. No. S-9765) and dimethyl sulfoxide (DMSO; Cat. No. D-8779) were obtained from Sigma Chemical Co. (St. Louis, MO). The high molecular weight fraction from chicory inulin was a kind gift from Raffinerie Tirlemontoise S.A. (Tienen, Belgium); Onion fructan (moderately branched FOS) and wheat stem fructan (highly branched FOS) were extracted from the appropriate source with hot water, decolorized with activated charcoal, and freeze-dried (7).

Guest edited as a special report on "Novel Dietary Fibers: The Importance of Carbohydrates in the Diet" by Barry McCleary and Ian Brown.

Corresponding author's e-mail: barry@megazyme.com.

### Measurement of Total Dietary Fiber

TDF was measured using AOAC Method **985.29**, with amyloglucosidase (Cat. No. E-AMGDF), thermostable α-amylase (Cat. No. E-BLAAM), and protease (Cat. No. E-BSPRT) from Megazyme. The method was performed exactly as described or with the following modifications:

(a) *Replacement of HCl with acetic acid for pH adjustment before amyloglucosidase addition.*—After incubation with protease, the pH was adjusted to 4.0–4.5 by addition of 5 mL 3M acetic acid (instead of 5 mL 0.561M HCl).

(b) *Incubation with fructanase.*—To remove high molecular weight fructan from samples containing fructan, 0.1 mL partially purified fructanase (Megazyme, Cat. No. E-FRMXLQ; 8) was added at the same stage in the procedure that the amyloglucosidase was added. Incubations were exactly as described for amyloglucosidase in the standard DF method.

(c) *Complete removal of RS.*—DMSO (15 mL) was added to the sample (1.000 g) in 400 mL tall-form beaker containing a magnetic stirrer bar. Beaker contents were stirred on a magnetic stirrer until the sample was completely dispersed. The beakers were covered with aluminium foil, placed in a shaking water bath at 95°–100°C for 10 min, and then removed and immediately placed on a magnetic stirrer. Tris-MES buffer solution (25 mL, 0.1M, pH 8.2) plus 0.1 mL thermostable α-amylase (E-BLAAM, 3000 U/mL) were added with stirring. The beaker was immediately covered with aluminium foil, returned to the shaking water bath, and incubated at 95°–100°C for 30 min. The sample beaker was removed from the water bath and cooled to 50°C. All further steps were as described in AOAC Method **991.43** for the measurement of TDF, except that incubations with protease and amyloglucosidase were both performed for 60 min (instead of 30 min) and at 50°C; after protease treatment, the pH was adjusted to 4.0–4.5 with acetic acid (5 mL, 3M) as described above.

### Measurement of RS

AOAC Method **2002.02** (9, 10) was routinely used.

### Measurement of Fructan

Three procedures were used to measure fructan and FOS; AOAC Method **999.03** (7, 11; enzymic-PAHBAH; Format 1); a second method based on the use of hexokinase, phosphoglucose isomerase and glucose 6-phosphate dehydrogenase to measure glucose and fructose (enzymic-UV; Format 2); and a third method (Format 3) in which reducing sugars are removed by borohydride reduction, followed by enzymic determination of glucose and fructose according to Format 2. Format 3 may prove optimal in situations where low levels of FOS or fructan need to be measured in the presence of high levels of sucrose and/or malto-oligosaccharides. All methods use a specific α-glucosidase (sucrase) to hydrolyze sucrose to glucose and fructose, and a highly purified exo-inulinase/endo-inulinase mixture to hydrolyze fructan to fructose and glucose. AOAC Method **999.03** (11) and Format 3 use borohydride reduction to remove glucose, fructose, and sucrose (after hydrolysis to glucose and fructose by sucrase). The enzymic-UV method (Format 2) measures glucose plus fructose in sample aliquots treated with sucrase followed by fructanase. This method is a modification of the procedure described by Andersen and Sorensen (12), and is described in detail below.

### Determination of Fructan and FOS Using a Hexokinase-UV Format (Format 2)

*Enzymes.*—(a) *Sucrase (100 U)/maltase (1000 U) as freeze-dried powder plus bovine serum albumin (BSA).*—Store desiccated at –20°C. Dissolve entire contents of vial in 11 mL Buffer 1 [sodium maleate (0.1M, pH 6.5)]. Divide into aliquots of appropriate volume and store frozen in polypropylene containers between use. Stable for >2 years at –20°C.

(b) *Fructanase.—Highly purified exo-inulinase (5000 U) and endo-inulinase (100 U) as freeze-dried powder.*—Store desiccated at –20°C. Dissolve contents of vial in 11 mL Buffer 2 [sodium acetate (0.1M, pH 4.5)]. Divide into aliquots of appropriate volume and store frozen in polypropylene containers between use. Stable for >2 years at –20°C.

(c) *Hexokinase (425 U/mL), glucose 6-phosphate dehydrogenase (212 U/mL), phosphoglucose isomerase (840 U/mL).*—2.2 mL in 3.2M ammonium sulfate plus stabilizer. Store at 4°C. Use as supplied. Stable for >2 years at 4°C.

*Cofactors*—(a) *Nicotinamide-adenine dinucleotide $(NAD)^+$ (150 mg) and adenosine triphosphate (ATP; 440 mg) as freeze-dried powder.*—Store at –20°C. Dissolve contents of 1 vial in 12 mL distilled water. Divide into appropriately sized aliquots and store at –20°C between use and on ice during use. Stable for ca 12 months at –20°C.

*Buffers.*—(a) *Sodium maleate buffer (100mM, pH 6.5).*—Dissolve maleic acid (11.6 g, Sigma, Cat. No. M-0375) in 900 mL distilled water and adjust pH to 6.5 with sodium hydroxide solution (2M). Adjust volume to 1 L. Store at 4°C.

(b) *Sodium acetate buffer (100mM, pH 4.5).*—Add glacial acetic acid (5.8 mL) to 900 mL distilled water. Adjust to pH 4.5 using 1M sodium hydroxide. Dilute to 1 L. Store at 4°C.

(c) *Tris/HCl (100mM) plus magnesium chloride (5mM) buffer (pH 7.6).*—Add 12.1 g Trizma Base (Sigma, Cat. No. T-1503) and 0.48 g $MgCl_2$ (anhydrous) to 900 mL distilled water, and dissolve by stirring. Adjust pH to 7.6 with 1M HCl and dilute to 1 L. Store in a well-sealed Duran bottle at 4°C between use. Add 2 drops toluene to prevent microbial contamination. Store at 4°C.

*Measurement of fructan.*—(a) *Extraction of fructan and FOS.*—Mill dry samples to pass 0.5 mm screen. Cut solid fatty samples (e.g., chocolate) into fine shavings with sharp knife; analyze soft food products (e.g., spreads) without further preparation. Before they are weighed, all samples should be at room temperature. For samples containing 0–12% fructan (plus sucrose control flour), accurately weigh 1.0 g sample into dry Pyrex beaker (100 mL capacity) and add 40 mL hot

distilled water (ca 80°C). Place beaker on hot-plate/magnetic stirrer, stir, and heat (at ca 80°C) for 15 min (i.e., until sample is completely dispersed). Allow solution to cool to room temperature, and then quantitatively transfer it to 50 mL volumetric flask and dilute with distilled water. Mix contents thoroughly. If sample contains significant quantities of glucose, fructose, and/or sucrose (ca 30–60%, w/w), aliquots will need to be diluted a further 5- or 10-fold before assay. Mix solution thoroughly and repeat assay.

For samples containing 12–100% fructan (or fructan plus sugars), accurately weigh ca 1 g sample into dry Pyrex beaker (800 mL capacity) and add 400 mL hot distilled water (ca 80°C). Place beaker on hot-plate/magnetic stirrer stir, and heat (at ca 80°C) for 15 min (i.e., until sample is completely dispersed). Allow solution to cool to room temperature, and then quantitatively transfer it to 500 mL volumetric flask and dilute to mark with distilled water. Mix contents thoroughly. Filter an aliquot of the solution through a Whatman No. 1 (9 cm) filter circle and analyze immediately. If solution is still turbid, filter an aliquot through Whatman GF/A glass fiber filter paper. If this solution is stored for several hours at low temperature before analysis, the fructan may tend to precipitate from solution. In such cases, the solution should be reheated to ca 80°C and allowed to cool to room temperature before samples are removed for analysis.

(b) *Hydrolysis of sucrose and low DP maltosaccharides.*—Accurately dispense 0.2 mL aliquots of solutions to be analyzed (containing ca 0.1–2.0 mg/mL fructan) into bottom of glass test tubes (16 × 100 mm). Add 0.2 mL diluted sucrase/maltase solution to each tube, and incubate at 40°C for 30 min. Add 0.5 mL, 100mM sodium acetate buffer (pH 4.5; Buffer B) to each tube with vigorous stirring on Vortex mixer. This is termed Solution A.

(c) *Hydrolysis of fructan.*—Accurately and carefully dispense 0.2 mL aliquots of Solution A (in duplicate) to bottom of plastic spectrophotometer cuvettes (3 mL volume, 1 cm lightpath). Add 0.1 mL fructanase solution [A] to bottom of 1 cuvette (reaction), and 0.1 mL 100mM sodium acetate buffer to second cuvette (blank). Mix contents thoroughly and cover cuvette with Parafilm® (American Can Co., Greenwich, CT). Incubate covered cuvettes at 40°C for 20 min in dry hot-block heater to effect complete hydrolysis of fructan to fructose and glucose (reaction cuvette containing fructanase enzyme).

(d) *Measurement of fructose plus glucose.*—Add 2.50 mL Tris-HCl buffer and 0.1 mL $NAD^+$/ATP solution to each cuvette, mix, and read absorbance of solutions ($A_1$) after ca 3 min. Start reaction by adding 0.02 mL hexokinase/phosphoglucose isomerase/glucose 6-phosphate dehydrogenase mixture to each cuvette. Mix cuvette contents with plastic spatula and incubate cuvettes at 40°C for 20 min. Read absorbance ($A_2$) at 340 nm. Determine absorbance difference ($A_2$–$A_1$) for both blank and reaction (fructanase treated). Subtract absorbance difference of blank from absorbance difference of reaction, thereby obtaining $\Delta A_{fructan}$.

(e) *Calculations.*—Calculate content of fructan plus FOS (%, w/w) in test sample as follows:

Fructan (%, w/w, as is):

$$\text{Fructan + FOS (g/100 g sample)} = \Delta A\text{fructan} \times F \times 5 \times 0.9/0.2 \times V \times 100/W \times 1/1000 \times 162/180$$

where $\Delta A$fructan = [($A_2 - A_1$) reaction] - [($A_2 - A_1$) blank]; F = conversion from absorbance values to μg fructose plus glucose = (100 μg fructose)/(absorbance value for 100 μg fructose); 5 = conversion from 0.2 mL as assayed to 1.0 mL; V = volume (mL) of extractant used (i.e., 50, 100, 200, or 500 mL); 0.9/0.2 = 0.2 mL was taken from 0.9 mL enzyme digest for analysis; W = weight (mg) of sample extracted; 100/W = factor to express fructan as percentage of sample weight; 1/1000 = factor to convert from μg to mg; 162/180 = factor to convert from free fructose and glucose, as determined, to anhydrofructose (and anhydroglucose), as occurs in fructan.

## Results and Discussion

### *Total Dietary Fiber*

Internationally, the generally accepted methods for measurement of dietary fiber are AOAC Methods **985.29** (4, 5) and **991.43** (6). These methods include enzyme treatment for starch and protein removal, precipitation of soluble DF components by aqueous ethanol, isolation, weighing of DF residue, and correction for protein and ash in the residue (13, 14). These methods evolved from the original proximate analysis system for feeds (15), through improvements and refinements (16–19). In 1935, Williams and Olmstedt (20) developed a more physiological method to estimate indigestible material. Their method simulated digestion by incubating the food samples with enzymes. Their work was the basis for enzymic gravimetric methods developed later by Hellendoorn et al. (21) and Thomas (22). Initially, AOAC Method **985.29** (4, 5) had some problems with reproducibility due to varying ash contents. This was due to formation of insoluble calcium phosphate from the phosphate buffer used and the high levels of calcium in the thermostable α-amylase then available. This problem was resolved by the introduction of Tris-MES buffer (AOAC Method **985.29**; 6) and by the availability of thermostable α-amylase containing low levels of calcium as stabilizer.

The limitations of AOAC Methods **985.29** and **991.43** became apparent over the past decade with the recognition that RS, which is incompletely analyzed by these methods, also behaves physiologically as dietary fiber. Furthermore, NDO, which now are also recognized as dietary fiber, were not measured at all. The recognition of RS and NDO as dietary fiber, has provided the opportunity for development of new fiber ingredients with properties that will ensure greater consumer acceptance and consumption of fiber. However, these developments have necessitated the concurrent development of specific methods for measurement of these new fiber components. For some materials, such as resistant starch and high molecular weight fructan, AOAC DF methods

**Table 1. Effect of addition of crude and purified fructanases on recovery of inulin and β-glucan in the AOAC total dietary fiber assay procedure (Method 985.29)**

| Enzyme preparation | Quantity, mL | Recovery, %[a] | |
|---|---|---|---|
| | | Inulin (fructan)[b] | β-Glucan[b] |
| Heat-treated fructozyme | 0.2 | 0.2 ± 0.2 | 11.4 ± 0.4 |
| | 0.1 | 0.2 ± 0.2 | 52.0 ± 2.0 |
| | 0.05 | 7.2 ± 0.3 | 80.0 ± 1.2 |
| Pure fructanase mixture | 0.2[c] | 0.2 ± 0.2 | 99.0 ± 1.3 |
| | 0.1 | 0.2 ± 0.2 | 100.8 ± 0.8 |
| | 0.05 | 3.5 ± 0.2 | 98.5 ± 1.8 |

[a] All samples were analyzed in duplicate.
[b] The amount of β-glucan and fructan used in assays was 100 mg.
[c] The recommended level of fructanase for use in assays.

**985.29** (4, 5) and **991.43** (6) measure some, but not all, of the component. Thus, for these materials, it is important to measure the component separately with a specific method, but also to completely remove all of the specific component in the analysis of DF by the AOAC methods, such that a proportion of the component is not measured twice.

In AOAC Methods **985.29** and **991.43**, before addition of amyloglucosidase, the pH is adjusted to 4.0–4.5 by addition of 0.561M HCl. Because none of the salt components have buffering capacity at pH 4.0–4.5, it is necessary to both check the pH after acid addition, and to correctly readjust it with NaOH or HCl. Replacing the 0.561M HCl with 3M acetic acid removes this uncertainty, as acetate buffers in the desired range and thus addition of 5 mL of 3N acetic acid to the sample slurry always gives a pH of 4.0–4.5.

### *Removal of High Molecular Weight Fructan*

In AOAC Methods **985.29** and **991.43**, some of the high molecular weight fructan is precipitated with other soluble DF on the addition of ethanol. Analytically, this causes problems if fructan is measured separately and then added to DF values. To resolve this problem, Quemener et al. (23) recommended the use of heat-treated Fructozyme® (Novozymes A/S Bagsvaerd, Denmark). The Fructozyme effectively depolymerized the fructan (meaning that it would remain soluble in 66% aqueous ethanol). The recommended heat treatment of the Fructozyme inactivated most of a pectinase enzyme present in the preparation (which would otherwise lead to underestimation of pectin); however, it did not inactivate β-glucanase (cellulase) in the preparation (24), and the level of cellulase was sufficient to give significant underestimation of β-glucan in samples (Table 1). This β-glucananse can, however, be removed chromatographically from the fructan degrading enzymes (exo-inulinase and endo-inulinase), and this preparation can be used to depolymerize fructan with no effect on β-glucan recoveries (Table 1).

### *Removal of RS*

Complete removal of RS in DF procedures requires complete solubilization of the starch before treatment of the sample with thermostable α-amylase. This can be achieved either by stirring the sample in cold 2M KOH or by incubating the sample in DMSO at 95°–100°C. Cold, 2M KOH gives effective solubilization, but subsequent enzyme treatment requires neutralization and, thus, the formation of high concentrations of salt. On addition of ethanol to precipitate soluble DF, high levels of salt also precipitate, leading to high and variable ash levels.

In contrast, incubating the sample in DMSO at 95°–100°C leads to minimum modification of other polysaccharides (e.g., pectins) and problems of salt precipitation are not introduced. A proposed format for introduction of DMSO pretreatment is shown in Figure 1. In the presence of DMSO, protease and amyloglucosidase are less stable at high temperatures; therefore, incubation with these enzymes is performed at 50°C (cf 60°C in AOAC DF Methods) and for 60 min (cf 30 min). Also, acetic acid is used for pH adjustment before amyloglucosidase addition.

The effect of DMSO treatment on determined DF values for a number of samples is shown in Table 2. The samples studied included a range of high amylose starches, potato starch, food samples, and pectin. For the high amylose maize starches [Hylon VII®, Hi-maize®, and Novelose 240® maize (National Starch and Chemical Co., Bridgewater, NJ), and CrystaLean® (Opta Food Ingredients, Inc., Bedford, MA)], AOAC Method **991.43** measures some of the RS as DF. However, if the values obtained are compared to resistant starch measured by a method that simulates in vivo conditions (AOAC Method **2002.02**; 9, 10), then the value match is quite variable. Clearly, for native, high amylose maize starch (Hylon VII), the value obtained by the DF method is much lower than that obtained by the RS method, whereas that for Hi-maize 1043 is higher with the DF method than with AOAC Method **2002.02**. In modified Method **991.43**, DMSO

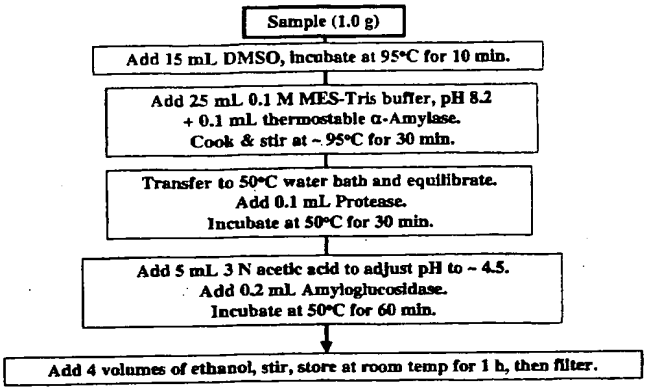


**Figure 1. Proposed scheme for introduction of DMSO into AOAC TDF Method 991.43.**

pretreatment is included, and RS is effectively solubilized and removed by subsequent α-amylase and AMG treatments.

ActiStar® (Cerestar, Vilvoorde, Belgium) and native potato starch granules give high RS values by AOAC RS Method **2002.02**. However, by the standard DF method, DF values are negligible. Native potato starch granules resist digestion in the small intestine, but are readily destroyed in boiling water. The same is true for Actistar, which is produced by controlled debranching of cassava starch by isoamylase (25). Clearly, AOAC DF Method **991.43** (and **985.29**) does not give true measures of the physiologically important DF content of these 2 starches. This also highlights the importance of determining RS values on samples "as eaten."

For a range of food samples, the DF values determined post-DMSO treatment (B) are lower than those without DMSO treatment. The difference in values (A–B in Table 2) is in quite good agreement with RS determined separately with AOAC RS Method **2002.02**. Clearly, for these samples, AOAC Method **991.43** does accurately measure the RS content of the sample (as part of TDF).

For a pure pectin sample, the determined DF value post-DMSO treatment is less than that without DMSO treatment. This possibly reflects the fragile nature of the pectin molecule and difficulties in obtaining quantitative precipitation with alcohol. With this material, incubations performed in the absence of any enzymes (and with no DMSO treatment) give DF recoveries of no more than 84–85%.

### *Measurement of RS*

By definition, RS is that portion of the starch that is not broken down by human enzymes in the small intestine. It enters the large intestine, where it is partially or wholly fermented. Consequently, any method used to measure RS must give values in line with those obtained with ileostomy patients. With this in mind, we reviewed most of the methods currently used for measurement of RS (26–31) and combined the better elements of each to develop a robust and reliable procedure that gives values in line with available physiological data. Because the term "resistant starch" relates to the physical nature of the starch rather than a defined chemical entity, basically, any values can be obtained for a particular sample, depending on the incubation conditions used. This is demonstrated in Figure 2, where the effects of time of incubation and incubation condition (shaking or

**Table 2. Total dietary fiber and RS contents of a range of samples**

| | Total dietary fiber[a] | | | RS |
|---|---|---|---|---|
| Sample | AOAC **991.43** (A) | Modified **991.43** (B) | Difference (A–B) | AOAC **2002.02** |
| Hylon VII | 25.9 | 1.0 | — | 53.7 |
| Hi-maize 1043 | 54.5 | 0.5 | — | 45.7 |
| Novelose 240 | 52.3 | 0.3 | — | 46.9 |
| CrystaLean | 34.0 | 0.3 | — | 40.9 |
| ActiStar | <0.1 | <0.1 | — | 58.0 |
| Native potato starch | <0.1 | <0.1 | — | 78.1 |
| Wheat bran | 38.7 | 38.0 | 0.7 | 0.42 |
| Hi-maize bread | 9.2 | 3.5 | 5.7 | 5.1 |
| Rye crispbread | 15.0 | 13.6 | 1.4 | 1.2 |
| Kidney beans | 21.5 | 16.5 | 5.0 | 5.3 |
| Corn flakes | 3.3 | 1.0 | 2.3 | 2.8 |
| Cooked/cooled potato | 7.1 | 5.4 | 1.7 | 3.8 |
| Pectin | 83.5 | 72.0 | 11.7 | 0 |

[a] Dietary fiber values determined by AOAC Method **991.43** and a modification in which the sample was first cooked with DMSO to solubilize RS. The difference (A–B) is RS measured by Method **991.43**, but removed by DMSO treatment.

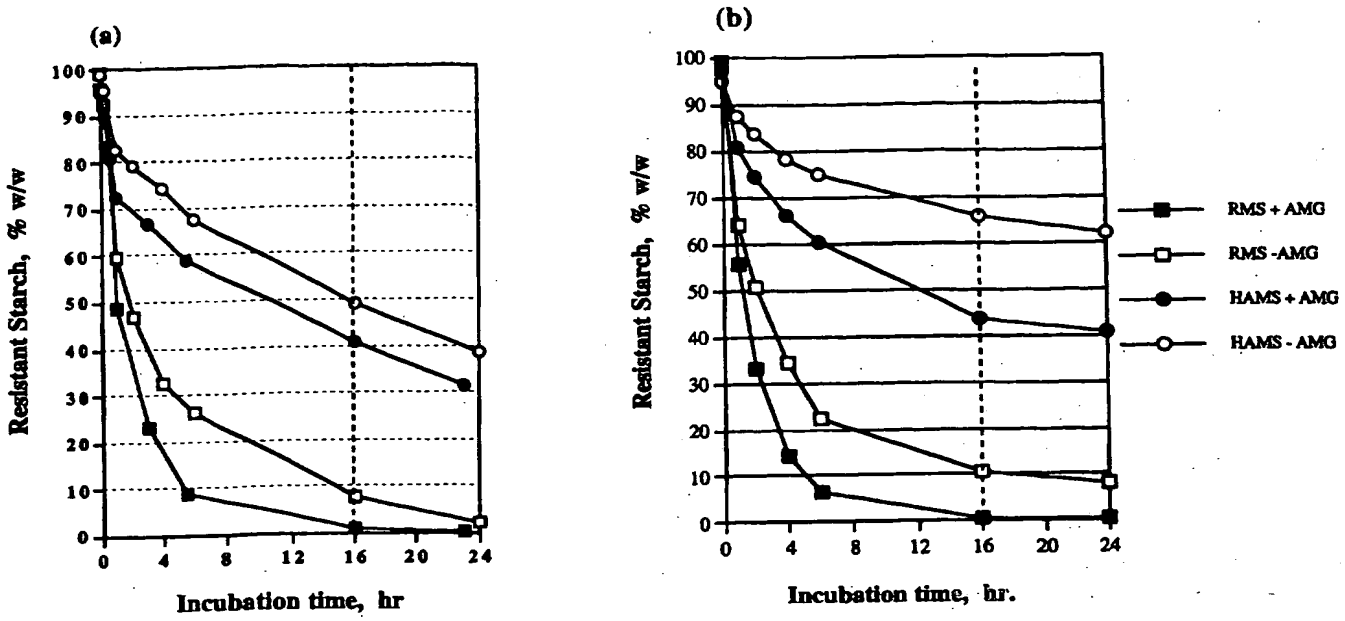


**Figure 2. Comparison of the effect of stirring and shaking on time course of hydrolysis of high amylose maize starch (HAMS) and regular maize starch (RMS) by pancreatic α-amylase (10 mg/mL) in the presence of AMG (12 units/assay; pH 6.0) or absence of AMG (at pH 6.9) at 37°C. (a) Tube contents continually stirred at 260 rpm with a magnetic stirrer bar; (b) tube contents continually shaken horizontally at 200 rpm.**

—stirring) are shown. Similar effects are shown with variations in the levels of amyloglucosidase and pancreatic α-amylase used and the pH of the incubations. In the current work, the conditions finally selected resulted in analytical results in line with those obtained from physiological studies (27; Table 3). The method developed was successfully evaluated in an AOAC interlaboratory evaluation (37 laboratories, 16 samples) to become AOAC Method **2002.02** (10).

Clearly, a validated, physiologically relevant method is required for standardizing RS measured in pure and applied research programs, and to act as a benchmark for evaluating new industrial ingredients and, potentially, for labeling

**Table 3. Comparison of RS values obtained using several in vitro analytical methods to in vivo results.**

| | RS (in vitro method/results)[a] | | | | | |
|---|---|---|---|---|---|---|
| Source of starch | Englyst | Faisant | Champ | McCleary | Goni[b] | RS (in vivo results) |
| Potato starch (native) | 66.5 | 83.0 | 77.7 | 77.0 | — | 78.8 |
| Amylomaize starch (native) | 71.4 | 72.2 | 52.8 | 51.7 | — | 50.8 |
| Amylomaize starch (retrograded) | 30.5 | 36.4 | 29.6 | 42.0 | 37.8[b] | 30.1 |
| Bean flakes | 10.6 | 12.4 | 11.2 | 14.3 | 15.3[c] | 9–10.9 |
| Corn flakes | 3.9 | 4.9 | 4.3 | 4.0 | 4.7[c] | 3.1–5.0 |
| Canned beans | 17.1 | — | 17.1 | 16.5 | — | 16.5 |
| ActiStar[d] | 63[d] | — | 57[d] | 58.0 | 57[d] | 54[d] |

[a] Values are presented as a percentage of the total starch content of the sample. All data except that of McCleary, Goni et al. (31), and values for ActiStar®, are from Champ et al. (27)

[b] From Goni et al. (31).

[c] From Goni et al. (31), calculating RS as a percentage of total starch, assuming a starch content for bean flakes of 40%, and for corn flakes of 70% (based on in-house results for similar materials).

[d] Results kindly provided by Bernd Kettlitz, Cerestar (Vilvoorde, Belgium), except for values by McCleary, which were produced in-house. The Englyst data were produced by Englyst Carbohydrate Services, Champ data at INRA (Nantes, France), and Goni data at Cerestar.

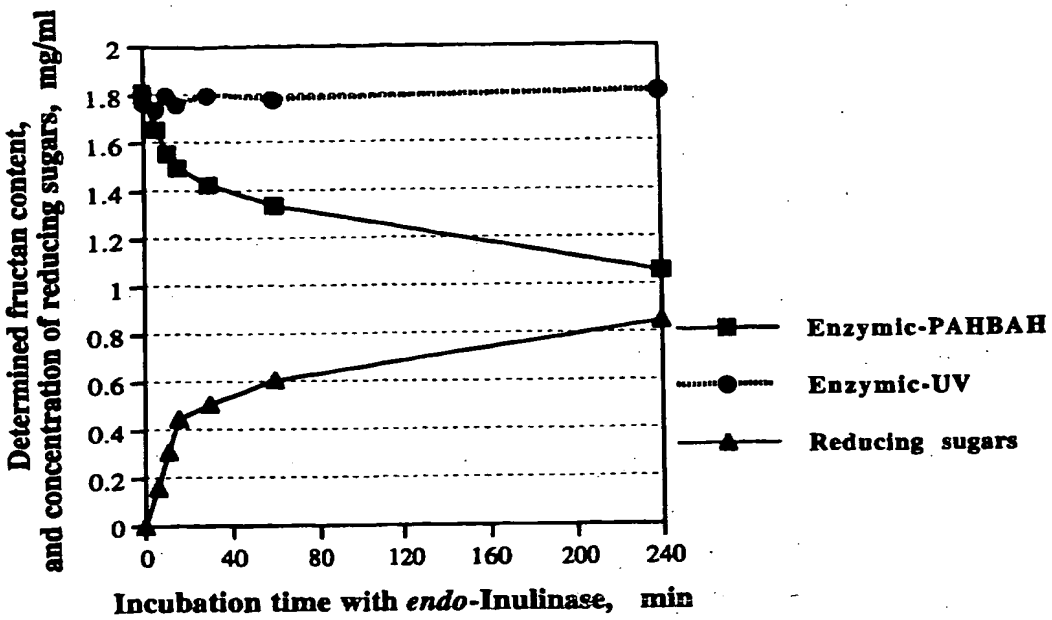


**Figure 3. Effect of hydrolysis of high molecular weight inulin on measured fructan content as determined by enzymic-PAHBAH and enzymic-UV methods. Raftaline (1 g) in 50 mL 100mM sodium acetate buffer (pH 4.5) was incubated with 50 U endo-inulinase at 40°C. Aliquots (5 mL) were taken at a range of time intervals up to 1000 min, and endo-inulinase was inactivated by incubation at 100°C for 5 min. Aliquots (10 μL) were applied to thin-layer chromatography plates, and samples were diluted and analyzed for fructan and reducing sugar levels.**

purposes. For most food samples, even those containing significant levels of starch, DF values obtained by the AOAC DF methods will suffice and accurately reflect the RS content of the sample (Table 2). However, in cases where high levels of RS are added as a food ingredient, then separate measurement of RS will be important to validate nutritional claims.

### *Measurement of Fructan and FOS*

Currently, there are 2 AOAC official methods for fructan measurement, an instrumental procedure (AOAC Method **997.08**; 32) and an enzymic-PAHBAH procedure (AOAC Method **999.03**; Format 1, described in Method; 11). The instrumental procedure requires the use of expensive equipment and is difficult to perform. The enzymic procedure, which involves specific hydrolysis of sucrose and removal through borohydride reduction, is simple to use, but suffers from the limitation that it underestimates the fructan content of partially hydrolyzed fructan. This problem is clearly demonstrated in Figure 3 and relates to the fact that, industrially, high molecular weight chicory fructan is subjected to controlled depolymerization by endo-inulinase to produce an ingredient (FOS) with more desirable ingredient properties (e.g., solubility). In the assay procedure for fructan, reducing sugars are reduced to sugar alcohols with sodium borohydride (so that these sugars will not be detected by the PAHBAH reducing sugar test). However, the terminal sugars at the reducing ends of hydrolyzed FOS are also reduced to sugar alcohols, meaning that these also will not be detected by the PAHBAH method. The conversion of the reducing-end D-fructosyl residue to a sugar alcohol may also render the terminal glycosidic bond more resistant to hydrolysis.

An alternative approach to the measurement of fructan in the presence of sucrose is to delete the borohydride reduction

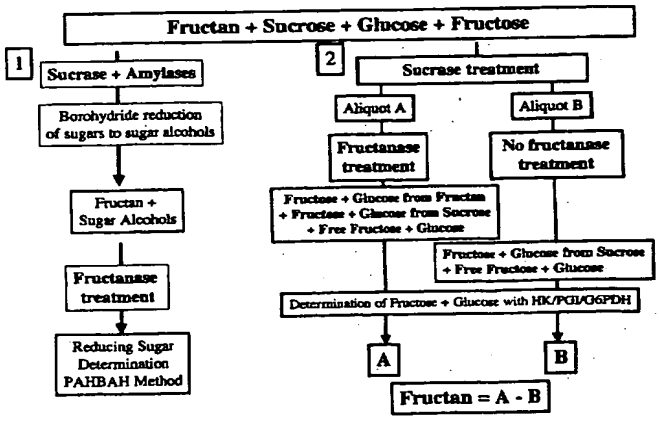


**Figure 4. Assay formats for measurement of fructan using enzymes; 1, the enzymic-PAHBAH procedure (AOAC Method 999.03); 2, the enzymic-UV method.**

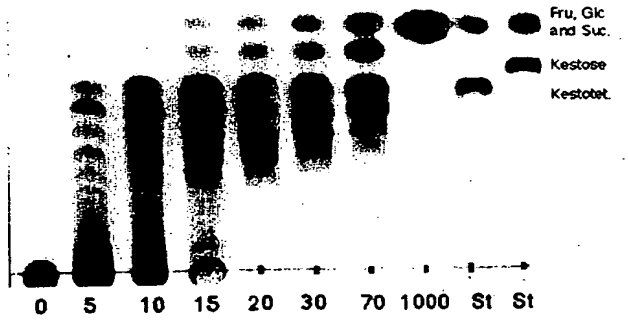


**Figure 5. Thin-layer chromatography (TLC) of oligosaccharides produced on hydrolysis of high molecular weight fructan with endo-inulinase. Incubation conditions are as described in Figure 3. Reaction samples (10 μL) were subjected to TLC (*n*-propanol–ethanol–water, 7 + 1 + 2). The TLC plates were developed once, and spots were visualized by spraying the plates with 5% sulfuric acid in methanol, followed by charring in an oven at 120°C (ca 5 min). Standard sugars are fructose, glucose, sucrose, kestose, and kestotetraose.**

step and to specifically measure glucose and fructose produced on complete enzymic hydrolysis of the fructan. Such a format (Format 2) is shown in Figure 4. AOAC Method **999.03** is shown in Format 1. In Format 2, the borohydride reduction step is removed. A sample of extract is incubated with sucrase enzyme, and aliquots are removed for direct measurement of glucose and fructose (free glucose and fructose in the sample, plus glucose and fructose from sucrose) or for treatment with fructanase to give fructose plus glucose from fructan, as well as the free glucose and fructose and the glucose and fructose from sucrose. Subtraction of the former from the latter yields glucose plus fructose from fructan and FOS.

The effect of partial hydrolysis of high molecular weight chicory fructan by endo-inulinase on measured fructan by the enzymic/PAHBAH method (AOAC Method **999.03**) and by an enzymic-UV method is shown in Figure 3. Clearly, as the degree of hydrolysis increases, fructan determined by the enzymic/PAHBAH method (Format 1) decreases, while that measured by the enzymic-UV method (Format 2) remains unchanged. Industrially, fructan is depolymerized to improve its solubility. In this process, it is also essential to minimize the formation of disaccharides (and monosaccharides) which, by definition, are not DF. A hydrolysis value of about 17–20% (achieved after 20 min incubation under the conditions shown in Figure 3) is optimal, which yields mainly oligosaccharides of DP 4–10 (Figure 5). The fructan content of this material determined by the enzymic-PAHBAH method is approximately 80–85% of that determined with the enzymic-UV method (i.e., approximately 20% underestimation).

The fructan contents of a number of samples as determined by the enzymic-PAHBAH and the enzymic-UV methods are shown in Table 4. For many samples, both methods give similar values, but for depolymerized fructan samples, the values are underestimated by the enzymic-PAHBAH method.

Although the enzymic-UV method does give quantitative measurement of fructan and FOS in samples, other problems are introduced as highlighted in Table 5. In this table, the absorbance values obtained by the enzymic-UV method (Format 2) for blanks (sucrase treatment) and reaction (sucrase plus fructanase treatment) are shown. Clearly, there are problems for materials containing high levels of glucose, fructose, and sucrose with low levels of fructan (e.g., chocolate plus Raftilose). The 2 absorbance values are quite high, leading to a substantial increase in experimental error. For such samples, the enzymic-PAHBAH method gives a negligible blank and thus much reduced errors but, for Raftilose P-95 (FOS), it also gives an approximate 15–20% underestimation of fructan content. For such materials, Format 3 (*see Method*) may prove optimal. In this format, sucrose and reducing sugars are removed via borohydride reduction, whereas the fructose and glucose from fructan are determined enzymically. A comparison of the blank absorbance values and final determined fructan contents for a selected range of samples is shown in Table 6. Clearly, borohydride reduction dramatically lowers the blank absorbance values (and thus associated errors). With industrial materials of known degree of hydrolysis (e.g., Raftilose P-95), a correction factor could be introduced into the calculations. For the routine introduction of a correction factor in the determination of a particular FOS material, it

**Table 4. Comparison of determined fructan contents of samples using the enzymic-PAHBAH (Format 1) and the enzymic-UV (Format 2) methods**

| Sample | Determined fructan content, %[a] (dwb)[b] | |
|---|---|---|
| | Enzymic-PAHBAH method | Enzymic-UV method |
| Raftiline HP (nonhydrolyzed inulin) | 99.4 | 98.4 |
| Neosugars | 94.0 | 96.9 |
| Inulin (dahlia) | 93.6 | 94.9 |
| Raftilose P-95 (enzymically hydrolyzed inulin) | 80.3 | 94.5 |
| Chocolate containing Raftilose P-95 | 5.6 | 5.4 |
| Milk powder containing Raftilose P-95 | 5.8 | 5.2 |
| Wheat tissue | 4.9 | 4.0 |
| Sucrose control (10.6% sucrose) | 0.18 | 0.12 |
| Fructan control (29% fructan) | 30.2 | 30.1 |

[a] Average of duplicate determinations.
[b] dwb = Dry weight basis.

**Table 5. Absorbance values obtained for blank and reaction solutions by the enzymic-UV (Format 2) method for a number of fructan-containing samples**

| Sample[a] | Absorbance at 340 nm[b] | | | Fructan content, % w/w (dwb) |
|---|---|---|---|---|
| | + Sucrase (A) | + Sucrase + Fructanase (B) | B–A | |
| Raftiline HP (nonhydrolyzed inulin) | 0.004 | 1.194 | 1.190 | 98.9 |
| Raftilose P-95 (enzymically hydrolyzed inulin) | 0.072 | 1.126 | 1.055 | 94.5 |
| Neosugars | 0.077 | 1.159 | 1.082 | 96.9 |
| Wheat tissue | 0.763 | 1.124 | 0.361 | 4.0 |
| Chocolate containing Raftilose P-95 | 0.570 | 0.671 | 0.101 | 5.4 |
| Milk powder containing Raftilose P-95 | 0.661 | 0.759 | 0.098 | 5.2 |
| Sucrose control (0% fructan) | 0.916 | 0.924 | 0.008 | 0.3 |

[a] For Raftiline, Raftilose, Neosugars, and the sucrose control (10.6% sucrose), approximately 200 mg sample (weighed accurately) was dissolved in 100 mL water and 0.2 mL was incubated with sucrase. For wheat tissue, chocolate and milk samples, 1 g was extracted with 50 mL water. Before analysis, the chocolate and milk powder extracts were diluted 5-fold in water.

[b] Average of duplicate determinations.

**Table 6. Effect of inclusion of borohydride reduction step on blank absorbance values (+ sucrase) and final fructan contents of a range of samples analyzed with the enzymic-UV method**

| Sample[a] | Absorbance at 340 nm[b] | | | Fructan content, % w/w (as is basis)[c] |
|---|---|---|---|---|
| | + Sucrase (A) | + Sucrase + Fructanase (B) | B–A | |
| **No borohydride reduction step (Format 2)** | | | | |
| Raftiline HP (nonhydrolyzed inulin) | 0.010 | 1.005 | 0.995 | 94.2 |
| Raftilose P-95 (hydrolyzed inulin) | 0.052 | 0.958 | 0.906 | 88.3 |
| Raftilose L-60 (hydrolyzed inulin) | 0.346 | 0.985 | 0.639 | 48.0 |
| Triflora (spread) | 0.675 | 0.900 | 0.215 | 20.7 |
| Chocolate cookies with Raftilose P-95 | 0.255 | 0.284 | 0.029 | 2.8 |
| Chocolate containing Raftilose P-95 | 0.325 | 0.424 | 0.099 | 5.4 |
| Candy containing Raftilose P-95 | 0.622 | 0.703 | 0.081 | 7.7 |
| Spaghetti containing Raftilose | 0.388 | 0.674 | 0.293 | 2.8 |
| **Including a borohydride reduction step (Format 3)** | | | | |
| Raftiline HP (nonhydrolyzed inulin) | 0.010 | 0.880 | 0.870 | 92.3 |
| Raftilose P-95 (hydrolyzed inulin) | 0.008 | 0.742 | 0.734 | 80.2 |
| Raftilose L-60 (hydrolyzed inulin) | 0.006 | 0.450 | 0.444 | 37.4 |
| Triflora (spread) | 0.027 | 0.963 | 0.936 | 19.8 |
| Chocolate cookies with Raftilose P-95 | 0.028 | 0.312 | 0.284 | 3.0 |
| Chocolate containing Raftilose P-95 | 0.020 | 0.540 | 0.520 | 5.6 |
| Candy containing Raftilose P-95 | 0.040 | 0.684 | 0.644 | 6.9 |
| Spaghetti containing Raftilose | 0.022 | 0.282 | 0.260 | 2.8 |

[a] For Raftiline, Raftilose P-95, and Raftilose L-60, approximately 200 mg sample (weighed accurately) was dissolved in 50 mL water and 0.2 mL was incubated with sucrase. For all other samples, 1 g was extracted with 50 mL water. Extracts were diluted according to the sugar and fructan contents of the samples before borohydride reduction to ensure that absorbance values were on scale.

[b] Average of duplicate determinations.

[c] Sample analyzed as received.

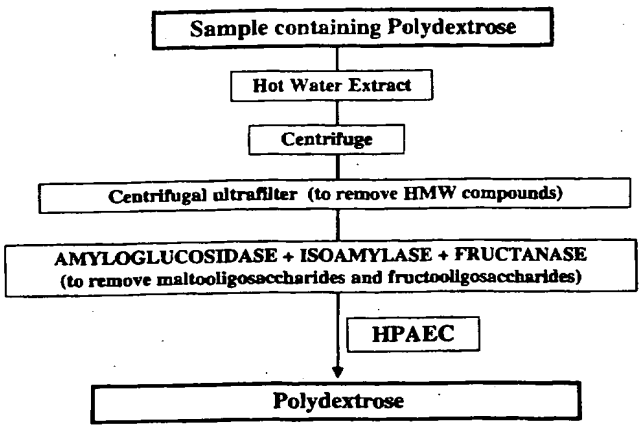


**Figure 6. Schematic representation of AOAC Method 2000.11 for determination of polydextrose.**

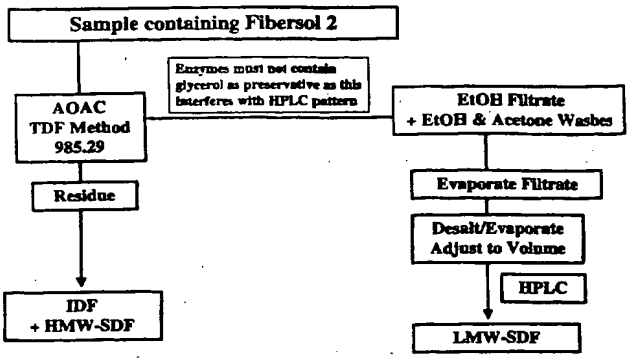


**Figure 7. Schematic representation of AOAC Method 2001.03 for determination of Fibersol 2. Fractions measured are insoluble dietary fiber (IDF) plus high molecular weight soluble dietary fiber (HMW-SDF) and low molecular weight soluble dietary fiber (LMW-SDF).**

would be essential that the degree of hydrolysis be known, and that this did not vary significantly between production batches. The degree of hydrolysis is readily determined by measuring fructan content of the original ingredient with Formats 2 and 3, and determining the "correction factor."

In summary, although all of the above assay formats measure fructan, each is limited to some extent by the nature of the sample being analyzed.

### *Resistant Maltodextrins and Galacto- Oligosaccharides*

Methods for the measurement of resistant maltodextrins (polydextrin and Fibersol 2) have been developed and subjected to interlaboratory evaluation. The procedure for polydextrose (AOAC Method **2000.11**; 33) is summarized in Figure 6 and that for Fibersol 2 (AOAC Method **2001.03**; 34) is shown in Figure 7. Both methods measure the particular analyte, but both also are relatively nonspecific. In the polydextrose method, treatment with amyloglucosidase, isoamylase, and fructanase is introduced to remove digestible maltodextrins and fructans but, potentially, other oligosaccharide materials could interfere with the assay. The method for Fibersol 2 also removes all digestible maltodextrins, but other oligosaccharides (e.g., FOS) which may co-occur in mixed food samples, will also be measured.

Galacto-oligosaccharides are specifically measured by a method (Figure 8) similar to that used to produce them. In this procedure (AOAC Method **2001.02**; 35), the sample extract is analyzed by high-performance anion exchange chromatography (HPAEC) for galactose and lactose. A separate sample is hydrolyzed by β-galactosidase and the hydrolysate analyzed by HPAEC to give galactose from galacto-oligosaccharides, plus galactose from lactose and free galactose. Subtracting the former from the latter yields galactose from galacto-oligosaccharides.

## Conclusions

With the recognition of NDO, inulin, and RS as DF, there has been a need for the development of methods to specifically measure these components. A method for the specific measurement of RS has been published and validated (AOAC Method **2002.02**; 10) and, in the current paper, we have proposed modifications to the AOAC DF methods to allow incorporation of these values. Although several methods have been developed for the measurement of NDO (some specific and some less specific) for food labeling and control purposes, there is a need for a universal method for measuring all NDO. Theoretically, this could be achieved with an liquid chromatgraphy (LC) method. Such a method has been used for measurement of Fibersol 2. However, a method in which oligosaccharides are extracted under milder conditions (e.g., 80°C with stirring) with the introduction of less buffer salts would be preferable. Extracts can be treated with amyloglucosidase plus sucrase at pH 4.5 to remove starch, maltodextrins, and sucrose, desalted and decolorized, concentrated (if necessary), and subjected to LC.

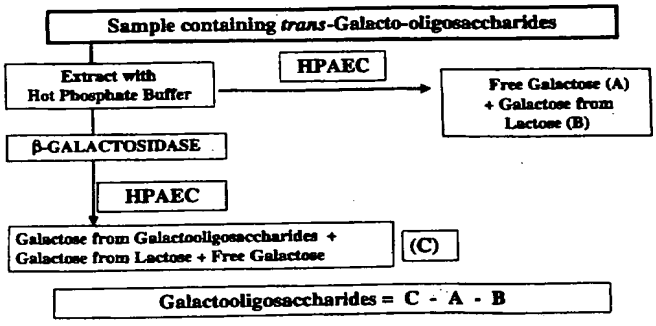


**Figure 8. Schematic representation of AOAC Method 2001.02 for determination of galacto-oligosaccharides.**

## Acknowledgments

The authors thank Richard Walker (BRI Australia Ltd.) for DF analyses.

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